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Title	Cell death, dendritic cells and downregulation of the immune response
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Qualification	PhD
Year	2003

Digitisation notes:

- Page 129 is used twice in the original thesis.

CELL DEATH, DENDRITIC CELLS AND DOWNREGULATION OF THE IMMUNE RESPONSE.

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Thesis presented for the degree of Doctor of Philosophy
University of Edinburgh
2002

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Edinburgh,
December 2002

To Adam

Abstract

Apoptosis is a critically important mechanism that facilitates deletion of unwanted or damaged cells in various circumstances including embryogenesis, inflammation and tissue healing. However, apoptotic cells are an important source of many autoantigens and the realisation that dendritic cells (DCs), the main antigen presenting cell of the adaptive immune system, not only internalise such dying cells but present antigen derived from them had important implications for our understanding of autoimmunity, tumour immunology and anti-viral responses.

The aim of this thesis was to explore the likely consequences of clearance of cells dying by constitutive apoptosis by myeloid phagocytes, with particular emphasis on the mechanism and outcome of DC clearance and the implications for autoimmunity. Firstly it will show that DCs generated from murine bone marrow demonstrate many characteristics attributed to DCs *in vivo* including endocytosis and phagocytosis, and mature upon receipt of danger signals such as endotoxin. However, internalisation of apoptotic cells does not augment DC maturation but rather inhibits subsequent responses to LPS, rendering these DCs less efficient than their neighbours at stimulating naïve T cells. These effects do not appear to be due to secretion of inhibitory cytokines such as TGFβ or IL10 and are not dependent on CD36 or β3/5 integrins, receptors thought to be involved in DC internalisation of apoptotic cells. In addition apoptotic cells inhibit LPS driven IL12 production by *ex vivo* DCs and in mice immunised with apoptotic cells *in vivo*. Furthermore, macrophages, likely to be found in high numbers in the inflammatory site also inhibit DCs and these inhibitory effects could be further augmented by the presence of apoptotic cells. Taken together these data demonstrate that internalisation of apoptotic cells by myeloid phagocytes modulates the adaptive immune response and suggest that the likely outcome of internalisation of cells dying by constituitive apoptosis will be tolerance rather than autoimmunity.

Acknowledgements

I have been fortunate to have three supervisors during my PhD and must thank each in turn; firstly, Jonathan Lamb, whose alternative view and imagination captured the magic of immunology; Adam Lacy-Hulbert, whose scientific integrity, rigour and desire to know the truth has been a driving force and finally, I must express my special thanks to John Savill for giving me this project in the first place, for his belief that it was worth doing and that I could do it, and for his wise counsel.

There are too many people who I have worked with to thank them all but I am specially appreciative to some; Yi Ren and Graham Thomas, who held my hand during the early days of my PhD, and whose departures left a big gap; Katy Hill, without whom the T cells would never have got off the ground; Shonna McColl, who taught me how to use a Gilson; Spike Clay, for making even the most onerous jobs entertaining; Aileen Smith, for always being there for everyone and Max Kremmel, for being interested enough in DCs to continue working on them. In particular I must thank Mark 'Sparky' Lucas with whom I have worked side by side and whose painstaking analysis of the macrophage has been a catalyst for many ideas.

One is only a product of past experiences and my family have a lot to account for; I must thank my sister for teaching me to write and my brother for sharing his love of science with me; my mother from whom I learnt never to take an idea for granted but to form my own opinions, and my father, whose numerous academic careers, showed me that your life can never be 'too' full. I must thank them all for their great senses of humor, for making every day an intellectual tour de force and for their shared intolerance of the mundane.

Finally, I cannot put into words my gratitude to my husband, for always listening, always understanding and always being the voice of reason – I think he, more than anyone, knows that this thesis was truly a labour of love.

This work has been funded by the Wellcome Trust.

Declaration

This work contains no material which has been accepted for the award of any degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

26/5/13

Lynda Maria Stuart Date

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

a.c.	Apoptotic cell
acMo	conditioned media from macrophages internalising apoptotic cell
AICD	Activation induced cell death
ALPS	Autoimmune lymphoproliferative syndrome
ANA	Anti-nuclear antigen
APC	allophycocyanin
CD	Cluster differentiation antigen
CMFDA	5-chloromethylfluorescein diacetate
CRP	C reactive protein
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-grabbing non-integrin
DMEM	Dulbecco's Modified Eagles Medium
dsDNA	double stranded Di-deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
ELISA	Enzyme linked immunoabsorbtion assay
EM	Electron microscopy
E.R.	Endoplasmic reticulum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GMCSF	Granulocyte monocyte colony stimulating factor
GMFI	Geometric mean fluorescence intensity
HLA	Human lymphocyte antigen
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MCM	Monocyte conditioned media
MHC	Major histocompatability complex
MoCM	Macrophage conditioned media

NK	Natural killer cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI	Propidium iodide
PPP	Platelet poor plasma
PS	Phosphatidyl serine
PsR	Phosphatidyl serine receptor
PRPDS	Platlet rich, plasma derived serum
SAC	Staphylococcus Aureus (Cowan strain)
SAP	Serum amyloid protein
SLE	Systemic lupus erythematosus
TAP	Transporters associated with antigen processing
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TLR	Toll like receptor
TSP	Thrombospondin
VnR	Vitronectin receptor
Vn	Vitronectin

PREFACE

"There is a big difference of course between natural death and accidental death, but basically death will come sooner or later. If from the beginning your attitude is, 'yes, death is part of our lives', then it may be easier to face.... The unfortunate event can be a source of inner strength" His Holiness the XIV Dalai Lama, 1995

Preface

"Dulce et decorum est pro patria mori" Horace ii.13, circa 65-8BC

I have never been greatly interested in the minutiae of cell death, be it apoptotic or necrotic. In all truth, as with life, all things come to an end and there is a certain inevitability about mortality. What is more interesting is the outcome of demise – is mourning inevitable or can we sometimes rejoice?

At a recent meeting someone asked of this work, 'why would our immune systems do that?' In other words what is the purpose of the anti-inflammatory response I have demonstrated here. This was interesting to me, coming from an inflammation lab where we have the perhaps bizarre focus, not on how inflammation is generated or maintained, but in how it is terminated. For this reason, I have, from the outset, been interested more in why things don't happen rather than why they do.

To answer this question I suppose we could start by considering the consequences of cell death. Apoptotic and necrotic cells do not languish *in vivo* but rather are cleared rapidly by any neighbouring cells, be they professional or semi-professional phagocytes. Thus one inevitable consequence of cell death appears to be phagocytosis. It has been suggested that this occurs for a reason truly innate - as a source of cellular nutrition. Through this 'self-cannibalism' multicellular organisms can recycle, regenerate and reuse precious nutrients especially in times of stress or need, such as during development and tissue remodelling.

Considering, therefore, that phagocytosis is as vital for recycling of fuel as for protection against pathogens, is it not likely that pathways involved in death, phagocytosis and response to infectious non-self would have evolved alongside one another. In certain circumstances, might it not be better if phagocytosis of dead cells continued without inciting a response, even when cell death was

caused by, or associated with, pathogen, and that only occasionally, if things were getting out of hand, would we require for the immune system to be activated? Thus anti-inflammatory signals from apoptotic cell death may well be dominant over conventional 'danger' signals.

An important point supporting this balance of power lies in the rarity of autoimmune driven disease, especially considering what we now know of apoptotic cells as a source of autoantigens. Although we could postulate that disastrous self reactivity or 'horror autotoxicous' does not occur because of 'lack of appropriate danger signals', 'lack of signal 2' or 'dose of antigen' these explanations leave us (and our immune system) in a rather precarious place, ready to be toppled by the first bout of flu or a developing mammary gland. Instead would it not be wiser to evoke active inhibitory or regulatory networks? And where else better for these to originate than with the apoptotic cell itself?

We now know that regulation exists at all levels of both the innate and adaptive immune system including macrophages, NK cells and T cells. Perhaps a more interesting question therefore, is how ancient are such regulatory networks? If, as suggested, phagocytosis, pathogens and cell-death have co-evolved will we find similar anti-inflammatory responses in our more primitive immunological ancestors? Some of our delay in answering these questions is simply experimental constraints – it requires us to devise novel ways of studying these 'negative' phenomena, redefine what we consider as 'immunity' to include these regulatory spectra and, of particular importance, to fully encompass the innate immune response. Without these modifications these areas will continue to remain overlooked.

CHAPTER 1: INTRODUCTION

1.1 Introduction

During normal tissue homeostasis, the rate of cell death is perfectly balanced by the rate of production of new cells resulting in a constant cell number (Kerr et al. 1972). Apoptosis is a critically important mechanism that facilitates deletion of unwanted or damaged cells in various circumstances including embryogenesis, inflammation and tissue healing. However, apoptotic cells are an important source of many autoantigens and the realisation that dendritic cells (DCs), the main antigen presenting cell of the adaptive immune system, not only internalise such dying cells but present antigen derived from them had important implications for our understanding of autoimmunity, tumor immunology and anti-viral responses.

The aim of this thesis was to explore the likely consequences of clearance of cells dying by constitutive apoptosis by myeloid phagocytes. DCs have been shown to play a vital role in linking the innate and adaptive immune response as well as being important semiprofessional phagocytes. For these reasons I chose to particularly focus on DC clearance and response to apoptotic cells and the implications for autoimmunity.

1.2 Autoimmunity

"The formation of tissue autotoxins would [therefore] constitute a danger threatening the organism more frequently and much more severely than all exogenous injuries" Paul Ehrlich

Autoimmunity occurs in 3-5% of the population and is characterised by pathology caused by, or associated with, ones own immune system. In these diseases the damage is often mediated either by self-reactive T cells or autoantibodies directed towards antigenic components of self-proteins. Autoimmune diseases are often associated with both genetic and environmental predisposing factors including HLA associations, susceptibility genes that control leukocyte reactivity and infectious triggers (Pickering et al. 2000a; Lesage et al. 2001; Marrack et al. 2001; Ohashi 2002). Autoimmunity is probably initiated by a single antigenic insult such as tissue destruction or infection, and, through determinant spreading, comes to involve either different parts of the same protein or other proteins within an organ. In many cases, such as multiple sclerosis and diabetes, autoimmune diseases are organ specific the target being determined by antigen expression, antigen presentation and vulnerability of the target organ. However, occasionally autoimmunity is directed at components of self that are expressed ubiquitously. In the archetypical systemic autoimmune disease, systemic lupus erythematosus (SLE), the antigens fall into two distinct types; either recently cleaved 'neoantigens,' such as those produced by granzyme B activity, or intracellular components, often found within multimolecular complexes such as dsDNA, ANA, chromatin and phospholipids (Rosen et al. 1999; Napirei et al. 2000). Interestingly, both the activity of granzyme B and the content of these multimolecular complexes is dependent on a process of cell deletion found commonly within inflammatory or remodeling tissue – 'apoptosis' (Casciola-Rosen et al. 1994; Rosen et al. 2001). The realisation that dendritic cells (DCs), not only internalised cells dying by apoptosis, but also present antigen derived from them (Bellone et al. 1997; Albert et al. 1998b) had important implications for many areas of immunology especially autoimmunity.

I chose therefore to study the fate of these dying cells and the DCs that engulf them, focusing on the specific implications for self-tolerance and autoimmunity.

1.3 Apoptosis and immune regulation

1.3.1 What is apoptosis?

Apoptosis is characterised by stereotypical morphological and biochemical changes including the activation of specific intracellular proteolytic enzymes (caspases) that cleave myriad nuclear and cytoplasmic substrates (Thornberry 1997). Apoptosis may result from an insufficient supply of survival signals or may be actively induced by various injurious stimuli such as hypoxia, reactive oxygen species, complement attack, nitric oxide, cytokines such as tumour necrosis factor-α (TNFα) or ligation of the Fas (CD95) cell surface death receptor (Nagata 1996). Apoptosis elicits specific cell surface changes, such as the exposure of phosphatidylserine (PS), normally found on the intracellular aspect of the cell membrane, resulting in the swift uptake and degradation of apoptotic cells either by local resident cells or infiltrating phagocytes (Savill et al. 1993; Savill et al. 2000b). This process is very rapid such that apoptotic cells are conspicuously absent in normal tissues. Furthermore, cell deletion by apoptosis leading to clearance by 'professional' phagocytes such as macrophages is not associated with proinflammatory mediator release (Meagher et al. 1992) but rather causes release of anti-inflammatory agents such as transforming growth factor β1 (TGFβ1) (Voll et al. 1997a; Fadok et al. 1998a; Fadok et al. 2000). The mechanisms whereby macrophages and 'semi-professional' phagocytes (including retinal pigment epithelial cells (Finnemann et al. 1999) and mesangial cells (Baker et al. 1994)) recognise and ingest apoptotic cells are complicated and may involve numerous molecules (Savill et al. 2000a) including the vitronectin receptor ($\alpha_v\beta_3$ integrin) (Savill et al. 1990; Savill et al. 1992; Rubartelli et al. 1997), CD36 (Savill et al. 1992; Albert et al. 1998a; Fadok et al. 1998c) thrombospondin , the phosphatidylserine receptor (Fadok et al. 1992a; Fadok et al. 1992b; Fadok et al. 2000), the first component of complement C1q (Taylor et al. 2000),

complement receptors (Mevorach et al. 1998a), class A scavenger receptors (Platt et al. 1996), CD14 (Devitt et al. 1998), calreticulin (Ogden et al. 2001) amongst others (Moynault et al. 1998; Oka et al. 1998; Imachi et al. 2000) (Fig 1.1).

1.3.2 The role of apoptosis in the maintenance of self tolerance and the regulation of T cell populations

Self-tolerance requires the removal or deactivation of autoreactive T cells with specificity to both central and peripheral antigens. Within the developing thymus engagement of autoreactive T cells by self-antigen induces apoptosis and deletion of these potentially injurious T cells. This process, referred to as central tolerance, depends critically upon apoptosis and consequently, defects in this 'programmed cell death' will facilitate the persistence of T cells capable of recognising self and inducing autoimmunity (Nagata 1996).

However, the entire repertoire of self-antigens is not represented within the thymus and therefore additional mechanisms are needed to maintain adequate peripheral self-tolerance (Walker et al. 2002). Although this process is incompletely understood apoptosis may also be involved. For example, it has been recognised for some time that certain organs such as the eye (Griffith et al. 1995; Griffith et al. 1996) and the testes (Bellgrau et al. 1995) are 'immunologically privileged' sites, relatively protected from the adaptive immune system. It is now apparent that this is at least partly due to the expression of Fas ligand by resident tissue cells at these sites. This tissue 'self defense' mechanism results in the induction of apoptosis in infiltrating Fas-bearing lymphocytes following Fas ligation (Singer et al. 1994a; Singer et al. 1994b; Bu et al. 2001).

Apoptosis also plays a role in the regulation of T cell populations. Clonally expanded populations of activated T cells that have served their functional purpose are rapidly deleted by 'activation induced cell death' (AICD), a process dependent upon ligation of the cell surface Fas death receptor

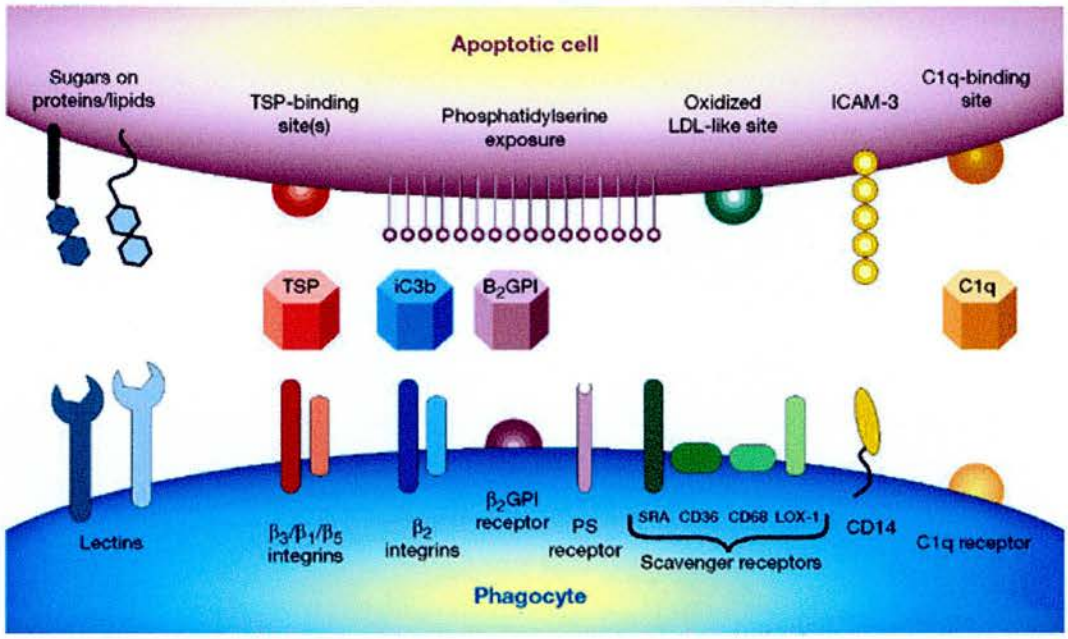


Figure 1.1: Apoptotic cells are recognised through a large number of apoptotic cell surface ligands, bridging molecules and receptors on the phagocyte surface. Taken from Savill and Fadok, 2000.

(Sytwu et al. 1996). Considering these three important roles for apoptosis in control of self-tolerance, it is therefore very pertinent that mice with mutations in the genes encoding either Fas or Fas ligand develop autoimmune disease (Nagata 1998). Depending upon the genetic background, such mice exhibit lymphadenopathy, splenomegaly, autoantibody formation, joint inflammation and glomerulonephritis with features of SLE (Cohen et al. 1991). Interestingly, early treatment of *gld/gld* mice, deficient in functional Fas ligand, with an agonistic anti-Fas antibody protected mice from the subsequent development of autoimmune disease by a mechanism which presumably involves Fas-dependent deletion of autoreactive lymphocytes. Furthermore, treatment of established autoimmune disease with the same agonistic anti-Fas antibody resulted in a significant improvement in disease pathology (Nishimura et al. 1997).

A small number of human patients have been described with mutations in the genes encoding either Fas or Fas ligand (Nagata 1998). These patients exhibit an autoimmune lymphoproliferative syndrome (ALPS or Canale-Smith syndrome) characterized by lymphadenopathy, splenomegaly and autoantibodies directed at blood components such as erythrocytes and platelets (Fisher et al. 1995; Martin et al. 1999). Affected individuals do not typically develop joint or renal disease and therefore defects in the Fas death receptor or its ligand do not appear vital to the pathology in these organs but can contribute to autoimmune disease. Taken together these observations support an important role for apoptosis in the deletion and removal of autoreactive cells.

1.4 Dendritic cells

"It is easy to be fascinated by dendritic cells, not only because of their pivotal role in the immune response but also because of the elegance with which they perform their tasks"
RM Steinman, 2001

1.4.1 DCs as innate and adaptive immune players

DCs are highly specialized antigen presenting cells and phagocytes, with important roles both in the innate and adaptive immune response (Banchereau et al. 1998; Liu, Y. J. et al. 2001b; Mellman et al. 2001). As innate immune players they efficiently internalise antigen and share many phagocytic and scavenger receptors with macrophages (Austyn 1996; Rubartelli et al. 1997). They are found well positioned, at portals of entry of pathogens such as the Peyers patches of the gut and in the skin, to perform their sentinel tasks. Besides a variety of endocytic receptors specialized for pathogen internalisation (e.g. the mannose receptor) they also have many pattern recognition receptors, including numerous members of the family of Toll-like receptors (Aderem et al. 2000; Kaisho et al. 2000), allowing them to recognize and respond directly to pathogen associated molecular patterns (PAMPS) (Reis e Sousa 2001), such as lipopolysaccaride (LPS), lipetechoic acid, dsRNA, CpG DNA or flagellin (Horng et al. 2001). In response to pathogens DCs secrete a variety of factors involved in early response effector functions (Huang, L. Y. et al. 2001; Rescigno et al. 2001) including IL12, type 1 and 2 IFNs and TNFα (Hochrein et al. 2001). These cytokines act to arm the effector cells of the innate response, such as NK cells, NK T cells and macrophages, against viral and bacterial pathogens as well as priming the adaptive immune response (Reis e Sousa et al. 1997). DCs can also be indirectly activated by environmental cues such as IL1 and TNFα, secreted by infected keratinocytes and epidermal T cells acting on bystander Langerhans cells (Yokota et al. 1996; Reis e Sousa 2001), and matrix components, such as hyaluronan (Termeer et al. 2002), and heparin sulfate (Kodaira et al. 2000). Furthermore, certain necrotic cells (Gallucci et al. 1999; Sauter et al. 2000), possibly via production of heat shock proteins (HSP) (Janetzki et al. 1998; Basu et al. 2000; Srivastava 2002b; Srivastava 2002a) or induction of proteolytic cascades, also provide suitable maturation stimuli for DCs.

The role of the DCs in adaptive immunity is even more subtle – qualitative and quantitative differences in their initial response contribute to determine the type

of the adaptive response that is initiated (Moser et al. 2000). For example, production of cytokines such as IL12, IL18 or IL10 can drive Th1, Th2 or Treg responses (Moser et al. 2000; Pulendran et al. 2001). This complexity may be added to further by different responses from different DC subsets (Shortman 2000; Liu, Y. J. 2001; Liu, Y. J. et al. 2001b) – CD8α+DCs produce large amounts of IL12 and prime Th1 responses and IFNγ production from interacting T cells; plasmacytoid DCs prime Th2 response and via production of type I IFN induce anti-viral immunity. This combination of responses and the functional consequences that ensue allow DCs not only to sense pathogen but also to activate the appropriate arm of the adaptive immune response, acting to bridge the innate and adaptive immune systems.

1.4.2 Immature vs. mature DCs and T cell interactions

It was proposed by Janeway that pathogens would be required to activate antigen-presenting cells, upregulating costimulatory molecules and T cell stimulatory function, for the immune system to be activated. Furthermore, he predicted the existence of pathogen associated molecular patterns (PAMPS) and a recognition system that would perform this function (Janeway, C. A., Jr. 1989b; Janeway, C. A., Jr. 1992). Matzinger further expanded this theory by suggesting that these 'danger' signals might be derived from endogenous products (Matzinger 1994). In support of this, DCs fulfill many of these requirements and appear to exist in at least two distinct activation states, immature and mature (Fig1.2) (Banchereau et al. 2000), controlled by exposure to 'danger' signals, be they exogenous or endogenous. Although this model probably oversimplifies the situation in vivo, it is useful to discuss for clarity.

In their immature state DCs are specialized for antigen capture, a task they perform by macropinocytosis, endocytosis and phagocytosis. In fact they

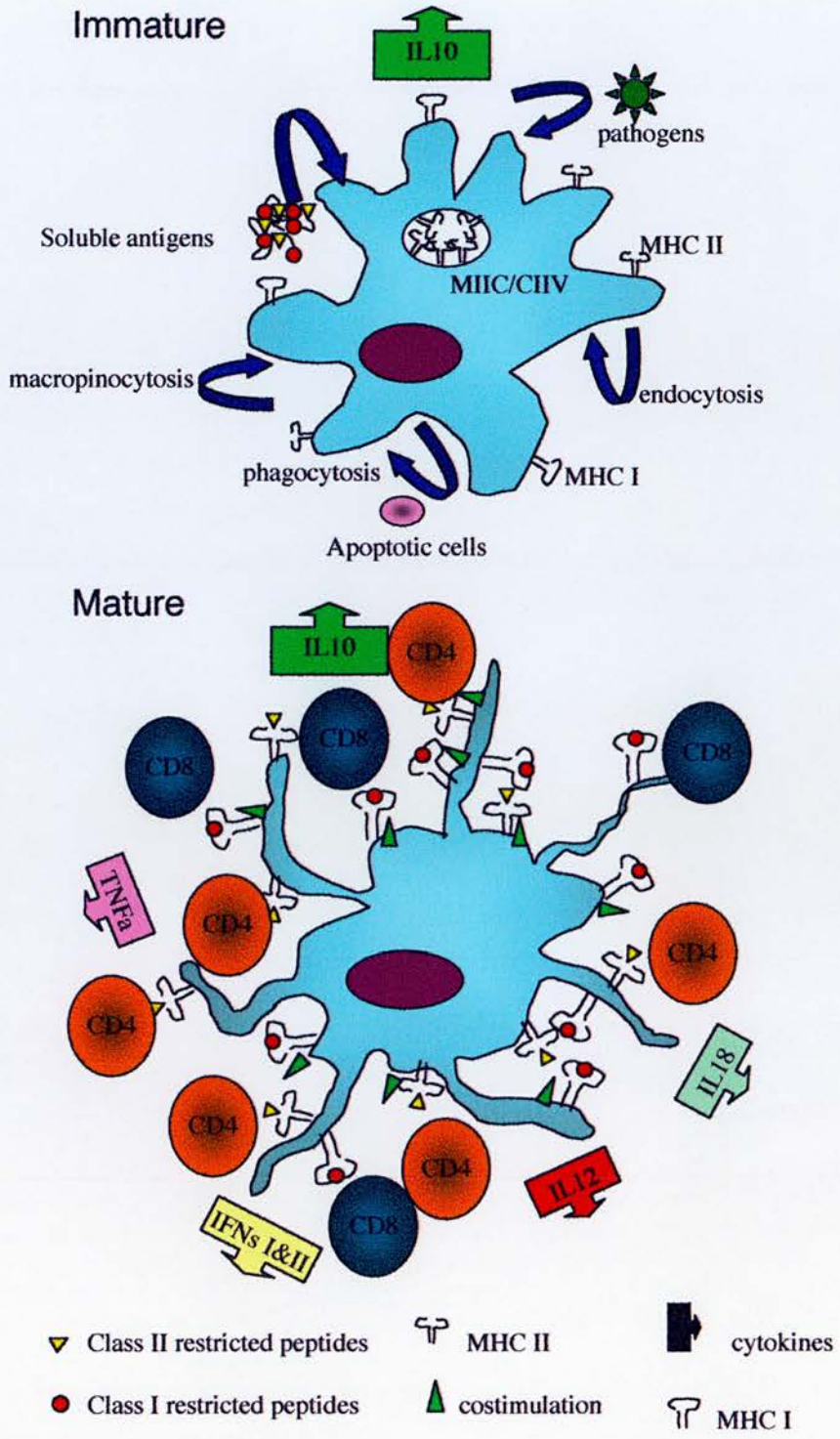


Figure 1.2: DCs exist in two distinct states, immature and mature

internalize such large volumes of antigen that they possess specific aquaporins for fluid egress (de Baey et al. 2000). Macropinocytosis and phagocytosis are more specific than endocytosis and require actin-cytoskeletal rearrangement, initiated after cell surface receptor ligation, for antigen internalization (Austyn 1996; Garrett et al. 2000; West et al. 2000). These mechanisms of antigen capture will be discussed in greater detail in chapters 3 and 6.

DC maturation is associated with movement of class II MHC from the lysosomal compartments to the cell surface, via class II rich vesicles (Mellman et al. 2001). In the mature DC the MHC molecules are expressed with increased stability (increasing from <10 to 100 hours) (Cella et al. 1997; Pierre et al. 1997; Inaba et al. 2000) and are found associated with increased levels of expression of costimulatory molecules on the cell surface (Caux et al. 1994; Inaba et al. 1994; Turley et al. 2000). Membrane rearrangement and up regulation of T cell adhesion molecules (including CD54 and DC-SIGN) also facilitate initial DC: T cell interactions and formation of the 'immunological synapse'. In addition DCs alter their chemokine receptor profiles, downregulating CCR5 and 6 and upregulating CCR7, allowing them to traffic to draining lymph nodes (Dieu et al. 1998; Yanagihara et al. 1998). Although initially thought that maturation and migration were linked it has been suggested that this is in fact not the case and that not all parts of the maturation programme need to be completed before DCs can exit the tissue (Randolph 2002). Finally, DCs produce a variety of cytokines (Langenkamp et al. 2000), able to modulate T cell responses, including IL12 (which is an absolute requirement for Th1 priming) (Magram et al. 1996). Thus DCs are unique in being able to provide all elements for T cell activation: 'signal 1' is provided by an abundance of MHC and stable MHC-peptide complexes, 'signal 2' as they express high levels of costimulatory molecules and 'signal 3' as they secrete the potent T cell activating cytokine, IL12 (Janeway, C. 1989a). Furthermore, the migratory properties of DCs allow both spatial and temporal compartmentalisation of DC functions; immature DCs acting in the periphery to capture antigen and mature DCs, having migrated to draining lymph nodes are

stimulated by T cells which provide signals, via ligation of CD40 (Cella et al. 1996; Schulz et al. 2000), inducing terminal maturation and differentiation.

1.4.3 Antigen processing and cross-priming

Antigens captured are targeted to specific lysosomal and endosomal compartments within the immature DC (Inaba et al. 2000; Turley et al. 2000). One such compartment is the late endosome in which large amounts of class II MHC is stored and it is within these specialised compartments that degraded peptides are loaded onto MHC II molecules (Fig1.3). In further support of Janeway's prediction, presentation of internalised antigen is not inevitable but instead requires an orchestrated maturation programme, initiated by TLR ligation, proinflammatory cytokines or CD40 (Cella et al. 1996; Schulz et al. 2000), before transportation of these MHC-peptide complexes to the cell surface occurs.

Bevan initially described cross-priming in the 1970s (Bevan 1976b; Bevan 1976a) when he observed that donor minor histocompatability antigens were found presented on recipient MHC. From this observation it was apparent that a recipient cell must internalise and present donor cell-associated antigens (Carbone et al. 1990). Unlike Class II MHC presentation of exogenous antigens, how class I is loaded with extra-cellular peptides remains poorly understood (Kurts et al. 1996) ((Norbury et al. 1997) and it is this latter phenomenon that is now usually called 'cross-priming' and appears unique to DCs (Heath et al. 2001a; Heath et al. 2001b).

Two distinct pathway of class I antigen presentation (cytosolic and endosomal) have been identified (Fig1.3). DCs are also unusual in that they sequester antigen in their lysosomal compartment whilst macrophages 'degrade' them. These antigens may well be able to 'leak' into the cytosol of the DC via a DC specific pore (Rodriguez et al. 1999a) or be directly 'injected' into the cytosol, as

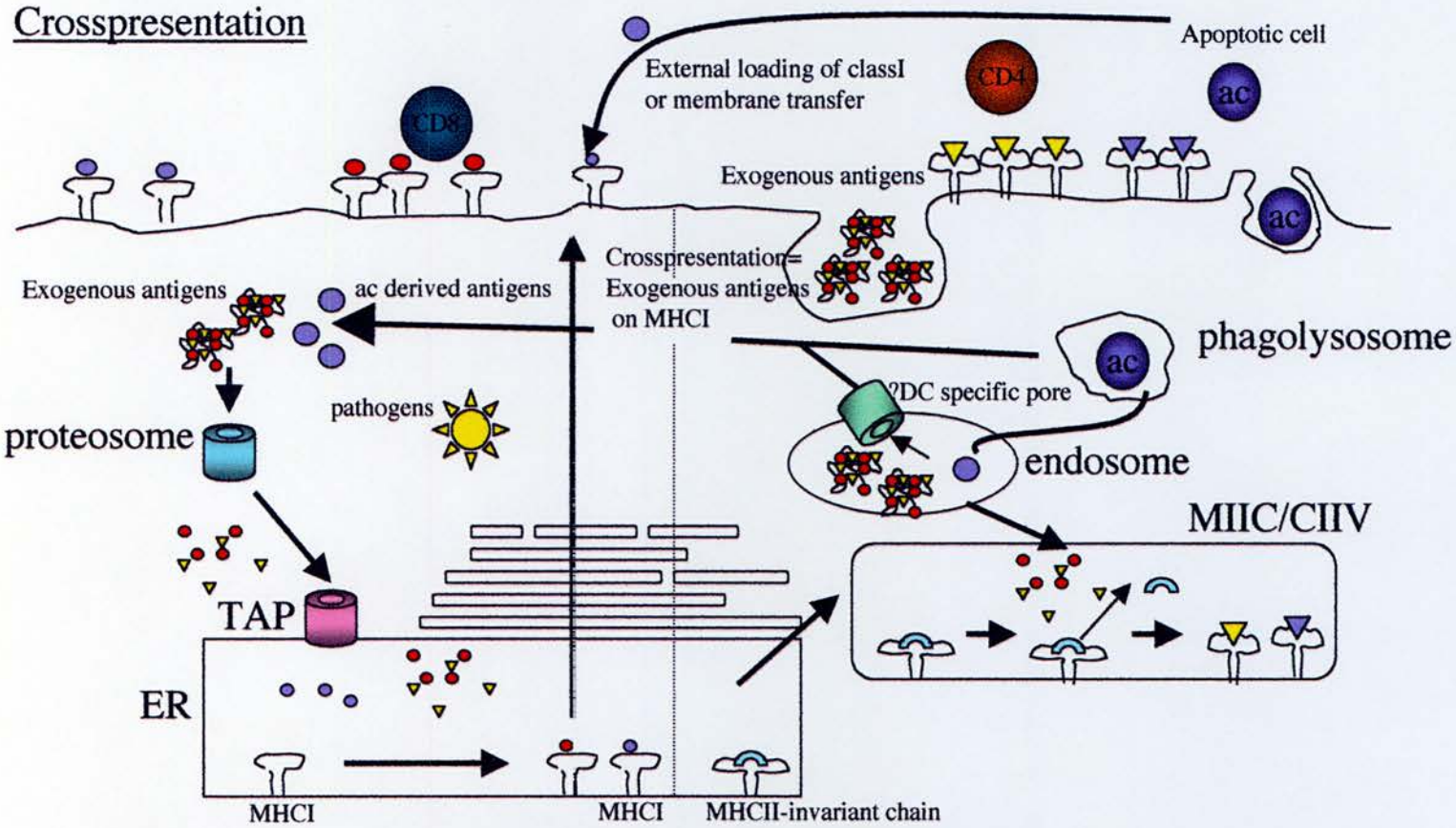


Figure 1.3 Pathways of antigen presentation. Adapted from Heath, 2001

is the case with infections by certain pathogens (Finelli et al. 1999). Here they are then available for class I loading via the conventional endogenous pathway and as such is TAP dependent (Huang, A. Y. et al. 1996; Norbury et al. 1997). Alternatively, class I restricted peptides may be presented independently of TAP (Bachmann et al. 1995; Schirmbeck et al. 1995) or be specifically chaperoned to the ER for loading (Srivastava 2002a), loaded directly at the DC cell surface where HLA-DM and class I may co-localise or presented from exosomes (Zitvogel et al. 1998; Thery et al. 1999; Wolfers et al. 2001) or extracted membrane from the cell of origin (Harshyne et al. 2001). In addition, apoptotic (Bellone et al. 1997; Albert et al. 1998a; Albert et al. 1998b) and necrotic cells (Lu, Z. et al. 2000) appear to be a highly efficient means of delivering exogenous peptides to both class I and class II MHC (Inaba et al. 1998) *in vitro* and are likely to be an important source of cellular antigens for cross-priming *in vivo*. However, cross-presentation is not a characteristic of all DC subsets and the identity of the cells responsible *in vivo* has remained elusive. It is only recently, nearly a quarter of a century after his original description, that Bevan has identified the CD8α+DC as a likely candidate (den Haan et al. 2000; Pooley et al. 2001).

1.4.4 DC subsets

Different functions of DCs can be explained not only by different maturation and activation states but also by separate developmental lineages with distinct functional attributes (Shortman et al. 2002). A detailed analysis of human DC subsets is beyond the scope of this thesis but bears many similarities to the murine subsets that have been described. To date, excluding plasmacytoid DCs (O'Keeffe et al. 2002a; O'Keeffe et al. 2002b), five murine DC subsets have been identified, characterised by cell surface markers and functional phenotypes (Anjuere et al. 1999; Vremec et al. 2000; Hochrein et al. 2001) with complex (and often contradictory) models of developmental origins (Liu, Y. J. 2001). Two important distinctions have been made by the expression of CD8α, the presence of which was initially thought to represent a specific DC lineage of lymphoid

origin. Although this division now appears to be an oversimplification CD8α+ DCs are still often referred to as lymphoid DCs and contribute about 25% of the DCs found within the spleen. These lymphoid DCs appear to have many important functional specializations such as *in vivo* IL12 production (Reis e Sousa et al. 1997) and biasing of Th1 response. In contrast, CD8α- (or 'myeloid') DCs make much less IL12 and preferentially drive Th2 responses (Maldonado-Lopez et al. 1999). Interestingly, CD8α+ DCs also appear to induce accelerated activation induced T cell death, which is not a feature of CD8α-DC: T cell interactions (Suss et al. 1996). Furthermore, CD8α+ DCs have been found to preferentially phagocytose apoptotic cells *in vivo* (Iyoda et al. 2002; Liu, K. et al. 2002; Schulz et al. 2002b) potentially explaining the efficiency with which they cross-present cellular derived antigens when compared to CD8α- DCs, which require an additional stimulus such as LPS to perform this task.

1.4.5 DCs and tolerance

Although exactly how peripheral tolerance is initiated and maintained is not fully understood it is likely that DCs are intimately involved - their ability to sample their surroundings, interact with naïve T cells and modulate the outcome of such interactions supports this suggestion (Steinman et al. 2000; Shortman et al. 2001). Certain DC populations such as the lymphoid DC may have distinct characteristics and contribute specifically to peripheral tolerance by anergising or deleting interacting T cells (Suss et al. 1996). Hence, the possibility that CD8α+DCs might cross-prime not only foreign or transplant antigens but also self-antigens is particularly pertinent as they may have the potential to delete a variety of self-reactive T cells. In support of this, deletion of interacting CD8 T cells appears to be the outcome of constitutive cross-priming *in vivo* (Kurts et al. 1997b; Adler et al. 1998; Morgan et al. 1999).

Another proposition is that it is not the DC subtype but its activation state that determines the induction of tolerance or immunity. For instance, immature DCs

which fail to express costimulatory molecules or IL12, carrying self-antigen (see later) may generate and maintain regulatory T cell subsets (Dhodapkar, M. V. et al. 2001). In a linear model of DC activation that has been recently proposed, early activated DCs might be specialized for production of IL12 and generation of Th1 response whilst later 'exhausted' DCs prime Th2 responses (Langenkamp et al. 2000). Finally DCs might be specifically modified by their tissue micro-environment, such as lung (Akbari et al. 2001) or gut (Iwasaki et al. 1999) DCs exposed to TGFβ or IL10 (Fiorentino et al. 1991; Steinbrink et al. 1997), providing particular cues and driving specific types of regulatory T cell responses.

1.5 Clearance of apoptotic cells and autoimmunity

1.5.1 Apoptotic cells express potential autoantigens near their cell surface

The origin of the autoantibodies typically present in the sera of patients with autoimmune conditions such as SLE (often directed towards ubiquitous intracellular antigens such as DNA, ribonucleoproteins and nucleosomes) has been perplexing as these autoantigens are normally 'invisible' to the immune system because of their localisation within the cell. However, seminal work by Casciola-Rosen et al indicated that keratinocytes undergoing apoptosis displayed potential autoantigens near their cell surface where they are available for interaction with immunologically competent cells (Casciola-Rosen et al. 1994; Rosen et al. 1999) (Fig 1.4). These potential autoantigens on apoptotic cells are likely to have undergone proteolysis by caspases and other proteases resulting in the production of immunogenic 'altered self' motifs (Napirei et al. 2000). Indeed, cleavage of intracellular substrates by the enzyme granzyme B can result in unique modification of potential intracellular autoantigens increasing their immunogenicity and raise the possibility that cytotoxic lymphocyte-mediated death of target cells (mediated by granzyme B) may play a specific

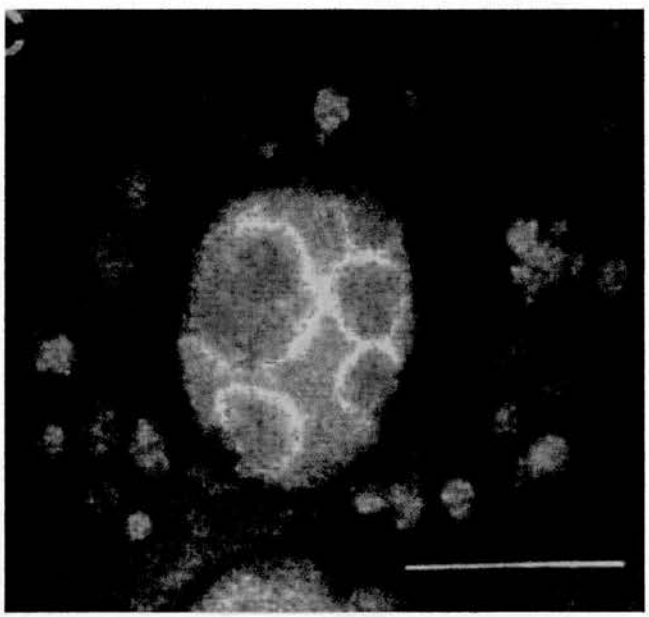


Figure 1.4: Apoptotic cells contain autoantigens. Apoptotic keratinocyte demonstrating co localisation of Ro (green) and DNA (red) to the surface of apoptotic cells and apoptotic blebs. From Casciola-Rosen et al (1994).

role in the development of autoimmunity (Rosen et al. 1999). It seems likely that the intracellular components such as dsDNA and chromatin will be found alongside neoantigens and 'altered self' motifs in macromolecular complexes on the surface of dying cells and may act as specific antigenic stimuli for generation and maintenance of self reactive B and T cells.

Despite the surface of apoptotic cells expressing potential autoantigens, it is obvious that the majority of the population does not develop autoimmune pathology and therefore apoptosis itself is insufficient to induce autoimmune disease. It is believed that, in normal circumstances, apoptotic cells are rapidly ingested and degraded by phagocytes. Indeed, *in vivo* studies of both renal development and glomerulonephritis indicate that the vast majority of apoptotic cells evident in tissue sections actually lie within other cells (Baker et al. 1994). This suggests that autoimmunity may be more likely to arise if there is a defect in the clearance of apoptotic cells and accumulating evidence suggests that this is indeed the case.

1.5.2 Evidence for an association of autoimmunity with defective apoptotic cell clearance

Experiments involving the injection of normal mice with irradiated syngeneic apoptotic thymocytes resulted in the transient development of antinuclear autoantibodies and anticardiolipin and anti-ssDNA antibodies, albeit at relatively low levels (Mevorach et al. 1998b). Furthermore, these mice also exhibited mild glomerular immunoglobulin deposition. These data indicate that exposure to large numbers of apoptotic cells, which may exceed the phagocytic capacity of the reticuloendothelial system, is able to elicit an autoantibody response.

As indicated previously, multiple macrophage cell surface receptors and bridging molecules may be involved in the recognition and ingestion of apoptotic cells. However, the involvement of C1q, the first component of the

classical complement pathway, is of particular interest since C1q deficiency is strongly associated with the development of SLE (Pickering et al. 2000b; Walport 2000). It is therefore extremely important that mice targeted for the deletion of the C1q gene spontaneously develop both autoantibodies and glomerulonephritis (Fig 1.5). Indeed, the glomerular inflammation in the *C1q-/-* mice is characterised by an impressive accumulation of apoptotic cells within glomeruli implicating defective clearance of apoptotic cells as an important aetiological factor in the development of disease (Botto et al. 1998). In addition, as in human disease, the genetic background of the Clq knockout mice plays an important role in modulating disease susceptibility and phenotype (Mitchell et al. 2002). A pathogenic role for defective apoptotic cell clearance in the development of autoimmunity may also explain why exposure to UV light or intercurrent infections are associated with increased disease activity since they would be predicted to increase the burden of apoptotic keratinocytes or leucocytes requiring phagocytic clearance. Lastly, it is pertinent that monocyte-derived macrophages isolated from patients with SLE exhibit reduced ingestion of apoptotic cells in a quantifiable *in vitro* assay of phagocytosis suggesting that a defect in apoptotic cell clearance may be relevant to the pathogenesis of SLE.

The receptor tyrosine kinase Mer has been shown to bind Gas6 homologue proteins bound to the phophatidylserine exposed on apoptotic cells. Similar to the *C1q-/-*, the *Merkd/Merkd* mice, bearing a tail deletion mutation in this receptor, also exhibit a defect in macrophage clearance of apoptotic cells and develop autoantibodies (Scott et al. 2001). Furthermore, examination of the Tyro-Axl-Mer triple knockout, in which all members of the receptor tyrosine kinase family are mutated, demonstrate not only autoantibodies but also develop a constellation of autoimmune pathologies including nephritis and arthritis (Lu, Q. et al. 2001).

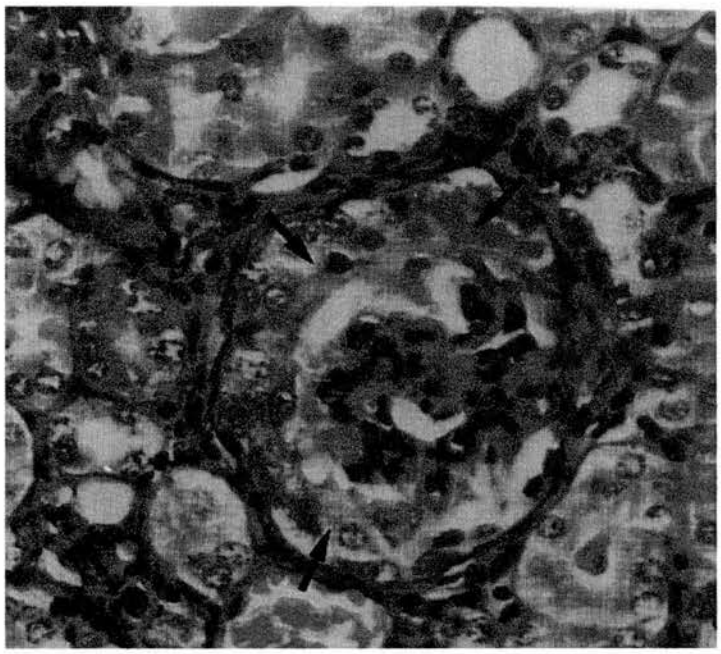


Figure 1.5: Defective apoptotic cell clearance is associated with autoimmunity. Glomerulus from *C1q-/-* mouse showing crescentic glomerulonephritis with free apoptotic bodies (arrows). From Botto et al (1998)

1.5.3 Evidence for mechanisms increasing safe clearance of apoptotic cells and preventing autoimmunity

Biological systems have evolved to ensure that apoptotic cells do not normally undergo secondary necrosis that would be predicted to increase the likelihood of autoimmune responses (Mevorach 2000). For example, C reactive protein (CRP), a teleologically ancient acute phase reactant protein, can bind to the apoptotic cell surface. Bound CRP stimulates activation of the classical pathway of complement resulting in opsonisation of the cell with complement components that can augment apoptotic cell clearance (Gershov et al. 2000) and also assists the maintenance of cell viability by inhibiting the activation of the cytolytic C5b-9 terminal membrane attack complex. In addition, the pentraxin PTX3, an acute phase protein generated locally within inflamed tissues, inhibits uptake of apoptotic cells by dendritic cells preventing presentation of apoptotic cell-derived antigen to T cells (Rovere et al. 2000). Lastly, the acute phase protein serum amyloid P component (SAP) binds chromatin on apoptotic cell surfaces as well as binding, solubilizing and regulating the degradation of free chromatin that results from cell breakdown. The importance of this chromatin scavenging system is underscored by the phenotype of mice targeted for the deletion of the SAP gene which exhibit antinuclear autoantibodies and glomerulonephritis (Bickerstaff et al. 1999).

1.5.4 Presentation of antigens derived from apoptotic cells

Unlike macrophages, dendritic cells are unique in their ability to stimulate primary immune responses. Immature dendritic cells exist in the periphery and constantly sample their antigenic milieu. In order to become fully functional antigen presenting cells they must mature and migrate to draining lymph nodes where they can interact with naïve T cells. Recent data indicate that dendritic cells not only ingest apoptotic cells but also are capable of presenting apoptotic

cell-derived antigen to cross-prime both CD4 and CD8 T cells if they receive appropriate activation signals (Bellone et al. 1997; Albert et al. 1998a; Albert et al. 1998b; Inaba et al. 1998). Since apoptotic cells are a source of potential autoantigens it is conceivable that dendritic cells internalising them might induce a primary autoimmune response. However apoptotic cells do not induce DC maturation and they would require receipt of concurrent 'danger' signals. Indeed, it has been suggested that a high load of apoptotic cellular material, perhaps due to secondary necrosis following failed clearance, may predispose to the development of autoimmune responses by increasing the potential for dendritic cells to phagocytose apoptotic cell material and subsequently present autoantigens (Kurts et al. 1998; Rovere et al. 1998; Ronchetti et al. 1999). However, autoimmunity is the exception rather than the rule and presentation of such apoptotic-derived self-antigens is likely to be tightly controlled. Hence, the ability of DCs containing apoptotic cells to respond to 'danger' signals is important and may be vital in modulating the outcome of cross-presentation of self-antigens derived from these dying cells, determining whether self-tolerance or autoimmunity ensues.

1.6 Conclusion

Apoptosis and the clearance of apoptotic cells are essential for normal tissue homeostasis, embryogenesis and tissue remodeling. Apoptotic deletion of potentially autoreactive T cells is involved in the maintenance of self-tolerance. However, apoptotic cells are an important and preferential source of many potential autoantigens and defective apoptotic cell clearance may facilitate the inappropriate presentation of these antigens by dendritic cells, thereby initiating autoimmune responses. In this thesis I will explore the likely outcome of DC interactions with cells dying by normal constitutive apoptosis. I will demonstrate that in normal circumstances regulatory effects, generated after phagocytosis of the apoptotic cells, in fact modulate DCs in ways which are

likely to contribute to prevention of autoimmunity and that presentation of apoptotic cell derived antigens will occur only in exceptional circumstances.

CHAPTER 2: METHODS

2.1 Animals

BALB/c mice were used for all experiments unless otherwise stated. Animals were purchased from B&K Universal (Hull, U.K.) and were used from 8 weeks of age. DO11.10 transgenic mice were breed in house at the University of Edinburgh animal facility and screened by tail bleed an FACS of peripheral blood lymphocytes. *CD36 -/-* were a kind gift of R. Silverstein and were breed in house at the University of Edinburgh animal facility and screened by tail tip PCR. *β3, β5* an *β3/5-/-* animals were a kind gift of K. Hodivala-Dilke and were breed and screen at the Institute for Cancer Research (Potters Bar) by tasil tip PCR and Southern. *IL10-/-* mice were purchased from Jackson Laboratory along with wild-type controls and used from 8-10 weeks of age.

2.2 Cell generation and cell culture

2.2.1 Bone marrow derived dendritic cells

Murine cells were cultured in RPMI 1640 supplemented with 2mM L-glutamine, 100U/ml penicillin, 100ug/ml streptomycin, and 10% heat inactivated foetal bovine serum unless otherwise stated. DC complete medium also contained 10-15% conditioned supernatant from a stably transfected B cell line (kind gift of Prof D Gray, Edinburgh) expressing recombinant GMCSF resulting in a final concentration of GMCSF of 20-30 ng/ml (Zal et al. 1994). This hybridoma also produces IL-10 at a final concentration of approximately 2ng/ml but no TNFα or IL12. All culture reagents were obtained from Gibco unless otherwise stated.
DCs were cultured as described previously (Lutz et al. 1999). Briefly, femurs from BALB/c mice were removed, dipped in 70% ethanol for 10 seconds and then placed in DC complete medium. Bone marrow was flushed from femurs and 10 ml of a single cell suspension of bone marrow cells at 2×10^5 / ml plated in non-tissue culture grade petri dishes. On day 3 a further 10 ml of fresh medium was added to the cultures. On day 6 half of the media was removed, and the cells pelleted, resuspended in fresh media and added back to the petri

dishes. On day 7 non-adherent cells were removed, leaving strongly adherent macrophages on the plate. These harvested cells were pelleted, re-suspended at 2 x 10^5 cells / ml and replated before use. On day 7 these were a heterogeneous population, 65-80% of the cells having surface phenotype and morphologyof immature DCs with granulocytes being the main contaminant. Maturation was initiated on day 7 with 0.1-1μg/ml of LPS (Sigma: *E. Coli* serotype 026:B6) or 0.02 % w/v *Staphlococcus Aureus* (Cowan strain) (SAC, Calbiochem) and cells assessed on day 8 (see Chap 3 for detailed analysis).

2.2.2 Bone marrow derived macrophage

Bone marrow was isolated as described for DCs. To generate macrophages cells were cultured in DMEM supplemented with 2mM L-glutamine, 100U/ml penicillin, 100ug/ml streptomycin and 10% heat inactivated foetal bovine serum and 10% conditioned supernatant from L929 cells, a source of murine MCSF. Cells were plated at 4x10^5 cells per well of a 24 well plate. On day 2 cells were washed and media changed. Macrophages were used for most experiments at day 6-7 of culture.

2.2.3 Isolation of human neutrophils

Neutrophils were extracted from peripheral blood of healthy volunteers as described previously (Savill et al. 1992). Briefly, blood was drawn into citrated 50ml Falcon tubes and spun at 350G for 20 minutes. Plasma was removed and to the remaining fraction was added 6ml of 6% dextran and tubes topped up with warmed normal saline before gently being inverted and allowed to sediment for 20 minutes. During this time a Percoll gradient was prepared. 100% Percoll was made up to 90% by addition of 4ml of 10xPBS to 36ml 100% Percoll. Using this 90% Percoll gradients were made from fractions containing 79%, 68% and 55% Percoll by addition of 1x PBS without Ca^{2+}/Mg^{2+}. The 2.5ml of the 68% fraction was overlayed onto 2.5ml of the 79% fraction in a 15ml

Falcon tube. The leucocyte enriched fraction was harvested from the top layer after the dextran sedimentation of the blood and pelleted at 350G for 6 minutes and resuspended in 2.5ml of 55% Percoll. This layer was overlayed onto the other two layers and the gradient spun at 720G for 20min (acceleration 9, deceleration 12, Sigma centrifuge). Peripheral blood mononuclear cells (lymphocytes and monocytes) were harvested from the top interface and peripheral blood neutrophils from the bottom interface. Cells were washed twice in PBS without Ca^{2+}/Mg^{2}before use. This yielded highly pure human neutrophils (>90 %). Autologous serum was made by clotting 10ml of plasma in glass tubes by addition of 200µl of 1M $CaCl_2$ at 37°C. Platelet poor plasma (PPP) was prepared by centrifugation of plasma at 3000rpm for 20 minutes.

2.2.4 Generation of apoptotic/late apoptotic and necrotic cells

Human neutrophils were allowed to undergo constitutive apoptosis by aging overnight in Iscove's medium (IMDM) supplemented with 100U/ml penicillin and 100ug/ml streptomycin and 10% autologous serum. After this period of time the cells were 40-80% apoptotic by cytospin morphology. This method of generating apoptotic bodies was preferred as there was no significant necrosis (<1%) by trypan blue exclusion, confirmed by annexin-PI staining and flow cytometry (Fig 2.1). Apoptotic murine thymocytes were also generated for use in some experiments by treating single cell suspensions of thymocytes with dexamethasone for 4-6 hours. This method yielded apoptotic cells but these preparations often contained contaminating post-apoptotic cells and other non-apoptotic thymic cells. For most experiments cells were stained using a green cell tracker dye (CMFDA) (Molecular probes) prior to overnight culture. To stain, neutrophils were suspended at high density ($20x10^6$ cells/ml) in serum-free IMDM and 10µl of 2mg/ml CMFDA added (to stain a maximum $120x10^6$ cells) for 15 minutes at 37C. Cells were then diluted out to $4x10^6$ cells/ml in IMDM with autologous serum.

Late apoptotic/apoptotic separation was performed on 24 hour aged neutrophils by density gradient centrifugation. To protect fragile late apoptotic cells, all procedures were performed at 4°C. Aged neutrophils were resuspended in plasma and overlayed onto a Percoll gradient made up of 31% Percoll (Percoll 620μl + 1380μl PPP), 42% Percoll (Percoll 840μl + 1160μl PPP) and 51% Percoll (Percoll 1020μl + 980μl PPP) in a 15ml Falcon tube. Gradients were spun at 550G for 20min at 4°C and cells harvested from the interfaces as follows:

Plasma/31% Percoll interface = pure late apoptotic cells

31%/42% Percoll interface = mixed late apoptotic and apoptotic cells

42%/51% Percoll interface= pure apoptotic cells

Necrotic cells were generated by heat treatment of live (primary necrotic) and apoptotic cell (secondary necrotic) at 100°C for 3 min.

Necrotic, apoptotic and late apoptotic cells were phenotyped using annexin-V and PI (Fig2.1)

2.2.5 Splenic DCs

DCs were isolated from spleen according to a modified protocol kindly provided by C Reis e Sousa (Reis e Sousa et al. 1997). Spleens were collected in RPMI and kept on ice before use. A single cell splenocyte preparation was obtained by digesting spleens using Liberase (Boehringer-Mannheim) at 1.67 WunschU/ml and DNAse (Boehringer-Mannheim) 0.2mg/ml in RPMI by injecting 5ml/spleen along length of the organ held over a 15 ml Falcon tube. The remaining spleen was dropped into the solution and left at 37°C for 30 minutes. Digested spleens were filtered through a cell strainer by rubbing the spleen onto the filter with the plunger from a 1ml syringe. Filtered cells were collected into 50 ml Falcon by washing with PBS/2mM EDTA. For most experiments the spleen suspension was further purified from debris by

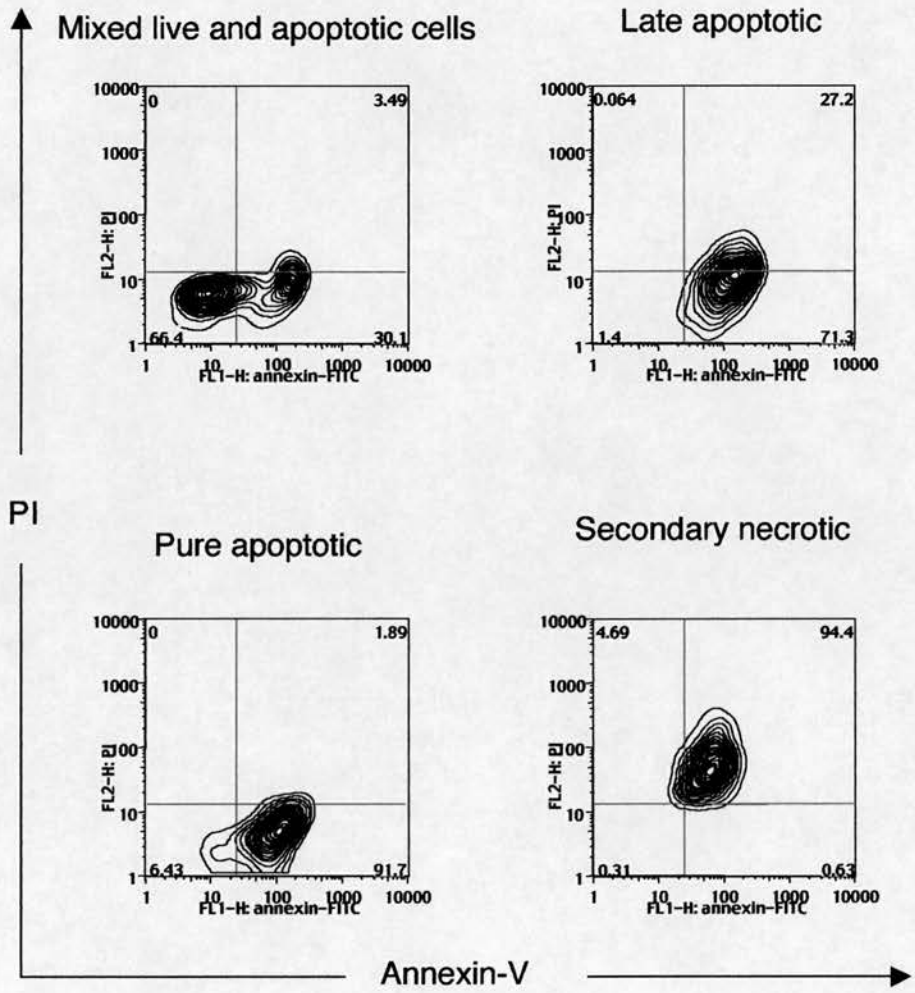


Fig 2.1 Surface phenotype of dying cells. Annexin-V/PI staining of 16 hour aged human neutrophils containing both live and apoptotic cells, purified apoptotic, purified late apoptotic and heat rendered necrotic neutrophils.

resuspending in 2x5 mls aliquots and spun through 5ml of Lympholyte-M (Cedarlane labs) at 1250G for 20 minutes according to manufacturers instructions, and cells isolated from the interface.

Cells were washed twice in PBS/2mM EDTA and resuspended in MACS buffer (100µl/spleen) left on ice for 2 min. CD11c-MACs beads were diluted 1:10 and 100 µl of diluted CD11c-MACS beads add 100 µl to each spleen to a final volume of 200ul. Cells were incubated in the fridge for 10 minutes, washed x2 in MACS buffer and resuspended in MACS buffer (in 0.5ml/spleen). Cells were passed through a 40µm strainer before adding to a positive selection column to yield 3-4X10^{6}DCs/spleen.

2.2.6 T cell isolation

CD4 T cells were isolated from spleens of DO11.10 transgenic mice. In brief, spleens were made into a single cell suspension by passing through a 53µm filter in PBS and debris and red cells removed by density gradient sedimentation through Lympholyte-M (Cedarlane Laboratories) according to manufacturer's instructions. Cells were then isolated using L3T4 (CD4) microbeads and MACS purification system (Miltenyi Biotec).

2.2.7 Hybridoma culture

L929 mouse fibrosarcoma line were cultured in Gibco DMEM/F12 supplemented with 10% FCS and 100U/ml penicillin, 100ug/ml streptomycin. Cells were passaged 1/10 2-3 x week. To harvest M-CSF containing supernatant, cells were grown to a confluent monolayer, media harvested and cleared by centrifugation.

GM-CSF was generated from a hybridoma (kind gift of Prof D. Gray). Hybridoma cells were cultured in Gibco IMDM supplemented with 100U/ml penicillin, 100ug/ml streptomycin, 2mM L-glutamine, 2Mercaptoethanol and

5% heat inactivated FCS. Selection antibioticG418 (Gibco-BRL 10131-019) was added at 1mg/ml and cells maintained between 7.5-40x10^4cells/ml by passaging 2-3 times per week 1:3 or 1:5. Cells were washed 3 times in media without G418 and resuspended in media without G418 at a medium density (i.e. 30-40x10^4 cells/ml) in 20 ml. Cells were grown in a 162 cm^2 flask to harvest supernatant. Media was harvested whilst still pink but cells thick and dense (0.75-1x10^6 cells/ml) and cleared by centrifugation before storing at –80°C.

2.3 Microscopy

All images were examined using an inverted microscope (Zeiss), and images captured using Open Lab software and CoolSnap digital camera unless otherwise stated.

2.3.1 Cytospins and Giemsa staining

70-100µl of cell suspension were spun at 400rpm for fresh cells or 300rpm for aged cells for 3 minutes onto glass slides. Slides were allowed to air dry, and then fixed in methanol 1 minute before staining. For Giemsa staining slides were stained in Diff-Quik 1 for 1minute, Diff-Quik 2 for 1 minute and washed with distilled water before mounting. For fluorescent microscopy cells were pre-stained before cyto-spinning and mounting in DPX under glass coverslips.

2.3.2 Plate based fluorescence

For fluorescent microscopy DCs were grown on chamber well slides (Lab-Tek), allowed to interact with red fluorescent apoptotic cells then fixed with 4% paraformaldehyde. Slides were stained in PBS with 0.5% BSA and 0.2% sodium azide with I-A^d-FITC (Pharmingen) in the presence of 10% normal mouse serum.

2.3.3 Electron microscopy

DCs that had been cocultured with apoptotic cells were pelleted and fixed with 25% glutaraldeyde (EM grade I) in 0.1 M sodium cacodylate buffer for at least 1 hour at room temperature. Fixative was removed with 1 wash of 0.1M sodium cacodylate buffer and the pellet imbedded in a resin plug. The plug was then processed with osmium tetroxide, lead citrate, araldite embedding and ultra thin sections (60nm) cut. Samples were analysed on a Philips CM150 TEM.

2.4 Flow cytometry

Cells were harvested, washed and resuspended in flow buffer. Non-specific antibody binding was blocked by preincubating cells with normal mouse serum for 10 minutes on ice. Cells were then pelleted at 300G for 2 minutes, resuspended in flow buffer and stained for 30 minutes on ice, in the dark. Antibodies were used at varying concentrations established by titration from 1 in 80 to 1 in 400. Some antibodies were not primary conjugated and hence a second step staining was required. Cells were then washed and resuspended in 200µl of FACS wash and analysed using FACSCalibur and FlowJo® software. The following antibodies were used (all from Pharmingen unless otherwise stated): FITC-I-A^d/I-E^d, PE-CD40, PE-CD86, PE-CD54, PE-CD11c, APC-CD11c, FITC-F4/80 (Serotec), unconjugated sialoadhesin (Serotec), unconjugated CD36 (Calbiochem), FITC-anti rat secondary (Serotec), FITC- anti mouse IgA (Pharmingen). All samples were compared to appropriate isotype controls. The geometric mean fluorescence of cells positive to isotype control was used in analysis (see Chap 3 for detailed analysis).

2.5 Phagocytosis/endocytosis assays

2.5.1 Flow cytometry based assays

Fluorescently labelled apoptotic cells were co-cultured with Day 7 DCs at a ratio of 2-5:1, apoptotic cells:DC. Most assays were performed in 96 well U-bottomed plates. Interaction of DCs with apoptotic cells was assessed by removing cells after 2 hours and staining with APC-CD11c for FACS analysis. All FACS analyses were carried out on a FACSCalibur flow cytometer (Becton-Dickinson). Gates defining phagocytosis were established by comparing cells co-cultured in the absence of fluorescent apoptotic cells (or, in some experiments, DCs co-incubated with apoptotic cells at 4°C) with DCs that had interacted with apoptotic cells at 37°C. Maximum interaction was seen at ratios of >5:1 apoptotic cells: DCs but the large numbers of uningested apoptotic cells reduced the efficiency of cell-sorting and so ratios of 2:1 were used in most experiments. Endocytosis assays were performed in a similar manner but the interaction was stopped by quenching with ice cold PBS and cells washed once with FACS buffer.

2.5.2 Cell sorting

To separate ac+ and ac- cells DCs were cultured with green fluorescent apoptotic cells for 2 hours at a ratio of 2:1. Cells were then activated using LPS and allowed to mature overnight (16 hours). DCs were next harvested and resuspended at 3-5x10^6/ml in RPMI with 10% FCS in sterile Falcon FACS tubes and sorted into ac^+ and ac^-DCs by the presence of forward and side scatter and coincident green fluorescence using a FACS vantage fluorescent cell sorter.

2.6 Cytokine assays

2.6.1 ELISA

ELISAs were performed according to manufacturer instructions (R&D). In brief, capture antibody was diluted in PBS and used to coat 96 well microtitre plates

by adding 100µl per well and incubating overnight. Each well was washed 3 times with ELISA wash buffer and then blocked using ELISA block solution for 1 hour. Plates were washed, loaded with 100µl of culture supernatant (or in the case of serum samples, diluted 1:100 in reagent diluent) and incubated for 2 hours. Plates were washed again and 100ml of detection antibody added for a further hour. Plates were washed and 100µl of streptavidin-HRP added for 20 min, washed and 100µl substrate solution added for a further 20 minutes before the interaction was stopped. Plates were read on an ELISA plate reader.

2.6.2 Intracellular cytokine staining

For intracellular cytokine staining, cells were co-cultured with apoptotic cells for 4 hours and stimulated with LPS for 5 hours in the presence of GolgiPlug (Pharmingen) 1µg/ml according to manufacturer's instructions. Cells were harvested and stained for cell surface markers as described above. Cells were then fixed using 4% paraformaldehyde and permeabilised with 2% saponin in PBS with 0.5% BSA and 0.2% sodium azide and 10% mouse serum whilst staining with APC-TNFα, IL10 and IL12p40/p70 (Pharmingen). Specificity of staining was confirmed by either preincubating with excess unlabelled antibody (IL12p40) or by addition of excess cytokine (TNF). All samples were compared to irrelevant isotype control and unstimulated cells.

2.7 T cell assays

2.7.1 Phenotyping DO11.10

8-week-old TCR transgenic mice were tail-bled 100µl in 500µl Alservers' solution in 1.5ml eppendorf tubes, topped up with PBS and kept on ice. Diluted blood was spun through 2.5ml of Lympholyte-M at 1250G for 20 minutes in FACS tubes. Lymphocytes were harvested form the interface. Peripheral blood mononuclear cells were stained for CD4 (Pharmingen) and KJ126 (Scottish Antibody Production Unit), a clonotypic antibody specific for the vβ8.2 T cell

receptor expressed as a transgene on DO11.10 T cells, to quantify percentage transgenic positive T cells. Both heterozygotes and homozygotes were used for experiments.

2.7.2 T cell stimulation

CD4 T cells were isolated from spleens of DO11.10 transgenic mice by Miltenyi MACS purification as described previously. Day 7 DCs were co-cultured with apoptotic cells for 4 hours, stimulated with LPS overnight and then were pulsed with 5μg/ml OVA peptide (OVA $_{323-339}$) (Albachem Laboratories, Edinburgh University) for 2 hour. They were then washed thoroughly and cells sorted into DCs containing apoptotic cells and those not, using a FACSVantage cell sorter (Becton-Dickinson). Contaminating apoptotic cells could be excluded from the sort by their smaller size and bright fluorescence. 3×10^5 OVA-TCR transgenic T cells were co-cultured in 24 well tissue culture dishes with varying doses of DCs for 5 days in a final volume of 2 ml. Proliferation was assessed by removing triplicate 100μl samples pulsed with 1 μCi / well of [^{3}H]- thymidine (Sigma) for 16 hours. Cells were harvested and thymidine incorporation measured using a scintillation counter. Interactions were performed in duplicate. For T cell restimulation T cells were washed, and resuspended in fresh media on day 5 of stimulation and allowed to 'rest' before being stimulated with PMA/ionomycin for 24 hours on day 7.

2.8 Miscellaneous

2.8.1 *In vivo* experiments.

Balb/C mice were used at 10 weeks. Apoptotic cells were washed twice with PBS and resuspended at 80×10^6 cells/ per ml and kept on ice. Mice were injected with iv endotoxin, 80 μg/mouse in 100μl of PBS. In addition 8x106 apoptotic cells were also injected either mixed with the LPS or subcutaneously.

After 4 hours the mice were sacrificed and bled by cardiac puncture. Serum samples were allowed to clot at 37°C for 1 hour, spun at 1500rpm and serum stored at –20°C until analysed by ELISA.

2.8.2 Annexin/PI staining

Apoptosis was assessed by cytospin morphology and annexin-V FITC and PI staining. For assessment of PS exposure cells were stained for 15 minutes on ice with annexin-V FITC in a Ca^{2+}/Mg^{2+} rich buffer. PI was used to assess membrane permeability by adding to FACS tubes at 0.1µg/ml for 120s prior to analysis.

2.8.3 Cell adhesion

Polylysine treated chamber well slides were either left uncoated or coated with matrix as follows: laminin, fibronectin and vitronectin was diluted to 10µg/ml, in 0.1M $NaHCO^3$, pH8, and 200µl added to each well. Wells were coated by rocking at room temperature for 3 hours before matrix was removed and the chamber wells used. Cell adhesion was performed by prestaining DCs using CMFDA as described for neutrophils and allowed to adhere to matrix coated plates for 1 hour at 37°C in the absence of serum prior to aspiration of excess media and addition of PBS. Cells were observed by fluorescent microscopy.

2.9 Statistical analysis

All data represents means +/- standard deviations unless otherwise stated. Data was analysed on Microsoft Excel using ANOVA statistical analysis. Differences were considered significant if $p<0.05$ and represented as '*' or highly significant if $p<0.01$ and represented by '**'.

2.10 Solutions, Reagents and tissue culture essentials

2.10.1 Solutions

PBS: 137mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH7.2-7.4
MACS buffer: PBS, EDTA(2mM), 0.5% BSA (TC grade), no azide
FACS wash: PBS without Ca^{2+}/Mg^{2+}, 0.2% BSA, 0.1% NaN_3
ELISA block solution: 1% BSA, 5% sucrose in PBS with 0.05% NaN_3
Reagent diluent: 1% BSA in PBS (0.2 μm filtered)
ELISA wash buffer: PBS/Tween (0.05% Tween 20 in PBS)
Sodium citrate: 0.1M Na Citrate, pH5
TMB: 10μg dissolved in 1ml DMSO
Substrate solution: sodium citrate, 2μl of 30% H_20_210ml, 75μl tetramthlybenzidine (TMB)/ per 10ml
Stop solution: 51ml dH_20 plus 3ml 2 N H_2SO_4
Alservers solution: 20g glucose, 0.55g citric acid, 8g trisodium citrate dehydrate, 4.2g NaCl made up to 1l with mH_20, pH6.1
Annexin buffer: Hanks balanced salt solution plus added 2mMCa^{2+}
Liberase: 250mg (1000 WunschU) bottle of CI lyophilised enzyme blend made up in 15ml sterile LPS free H_2O left on ice 30 min then filtered through a low-protein binding 0.22μm polysulphone membrane (eg Millipore Millex-GP syringe filter) (not nylon or nitrocellulose). 125μl aliquots were stored at -20-80°C

2.10.2 Reagents

Sigma:
Bovine serum albumin Fraction V #A9418
Sodium azide #S 8032
Citric acid #C7129
Polyoxyethylene-sorbtan monolaurate (Tween 20) #P1379
Saponin #S4521
Sterile DMSO #D2650
FITC-Dextran 70kD #FD70
FITC-Dextran 4kD #FD4

Cholera toxin #C8052

Pertussis toxin #P7208

LPS *E. Coli* serotype 026:B6 # L 8274

TMB #T2885

Latex beads #L0905

Vitronectin (rat) #83380-82-9

PI #P1470

Other:

Percoll Pharmacia

Dextran Pharmacia

Sucrose #BDH 10275C

Phosphate buffered saline tablets (Dulbecco's) #BR0014G

Pansorbin ® cells (lyophilised) Calbiochem

Dil-OxLDL Intracel #RP173

Dil-AcLDL Intracel #RP078

TGFbSR R&D # 41-R2

IL10 SR R&D # 474-MR

GRGDSP (Gly-Arg-Gly-Asp-Ser-Prol) Calbiochem #03-34-0035

GRADSP (Gly-Arg-Ala-Asp-Ser-Prol) Calbiochem #03-34-0052

GRGDTP (Gly-Arg-Gly-Asp-Thr-Prol) Calbiochem #03-34-0055

Fibronectin (murine) Gibco-BRL #12173-01

Diff-Quik I and II Dade Behring #130834/5

DPX mountant BDH #36029 4H

Laminin (natural mouse) Gibco-BRL #23017-15

Liberase Boehringer-Mannheim #1-814-435

DNAseI Boehringer-Mannheim #1-284-932

CMFDA Molecular Probes #C- 2925

Annexin-FITC Boehringer-Mannheim

2.10.3 Tissue culture reagents

Gibco-BRL:
RPMI 1640 #31870-025

DMEM (F12) # 21331-012

Geneticin (G418) # 10131-019

2 Mercapto-ethanol #31350-010

Sigma:
Dulbecco's PBS #D8537

EDTA Molecular Biology Grade # E7889

Bovine serum albumin 30% # 8327

Tissue culture plastics

Costar:
U-bottomed 96 well plates #3799

12 well plates #3513

24 well plates #3524

75cm^2 vented flask

162cm^2 vented flask

Other:
Falcon bacterial grade 10cm dishes #351029

0.5µm tissue culture inserts B.D Labware

Chamber well slides #154526

CHAPTER 3: EXAMINATION OF THE ENDOCYTIC AND PHAGOCYTIC FUNCTION OF DENDRITIC CELLS

3.1 Introduction

Dendritic cells have been described as the sentinels of the adaptive immune response. In an immature state in the periphery they capture antigen by macropinocytosis, receptor mediated and non-receptor mediated endocytosis and phagocytosis. Although DCs were known to be highly phagocytic they were only recently shown to be capable of internalising apoptotic cells and at the outset of this work few descriptions of this existed – two describing DC phagocytosis of apoptotic cells in a human system (Rubartelli et al. 1997; Albert et al. 1998a) and another in a murine DC cell line (Rovere et al. 1998). Thus, the first objective was to reproducibly generate murine bone marrow derived DCs and confirm their ability to internalise antigens including apoptotic cells. In addition it was necessary to establish a reliable system for evaluation of phagocyte : apoptotic cells interactions.

The data presented in this chapter demonstrates that murine bone marrow could be reliably differentiated into cells with cell surface, morphological and phenotypic characterisitics of DCs (Inaba et al. 1992a; Inaba et al. 1992b; Winzler et al. 1997; Lutz et al. 1999). In their immature state, these cells could capture antigen by endocytosis and phagocytosis and internalised antigen could be visualised by microscopy and reliably quantified by flowcytometry. This latter technique will allow, in future chapters, study of quantitative differences in cell surface expression of costimulatory molecules and cytokine production by individual DCs that had (ac+DCS) or hadn't (ac-DCs) phagocytosed apoptotic cells.

3.2 Results

3.2.1 Microscopy of DC and macrophage.

DCs and macrophages demonstrate strikingly different characteristics in culture. Macrophages grown in tissue culture plastic were strongly adherent laying down large amounts of extracellular matrix and growing, over 7 days, to a confluent monolayer (Fig 3.1). Removal of such cells was difficult requiring forceable washing and ice cold EDTA. In contrast DCs were only loosely adherent to plastic, growing mostly in grape like clusters. Phase contrast of such cells revealed irregular veiled protrusions and dendrites, characteristics from which the names 'veiled cell' and 'dendritic cell' were derived (Fig 3.1). Cytospins of macrophages and DCs also demonstrate morphological differences; macrophages were heterogeneous with a vacuolated cytoplasm (Fig 3.2) whilst DCs were extremely heterogeneous. Unfortunately, the fine veils and dendrites are difficult to preserve on cytospin morphology but can be seen on some cells (Fig 3.2).

3.2.2 Surface phenotype of DC *vs* macrophages

Although many of the surface characteristics of macrophages and immature DCs are similar, DCs can be readily distinguished from macrophages by the their ability to upregulate costimulatory molecules and class II MHC, which are rapidly mobilised from intracellular stores to the cell surface upon activation and maturation. Thus, to establish the phenotype of our murine bone marrow DCs, immature DCs, LPS matured DC and macrophages were compared for cell surface expression of a variety of myeloid markers (Fig 3.3). On day 7 immature murine DCs cultured according to the method of Lutz (Lutz et al. 1999) were 60-80% pure DCs as assessed by high expression of the relatively DC specific marker, CD11c although this is also expressed at lower levels on other myeloid cells. The contaminating cells at day 7 of culture were predominantly

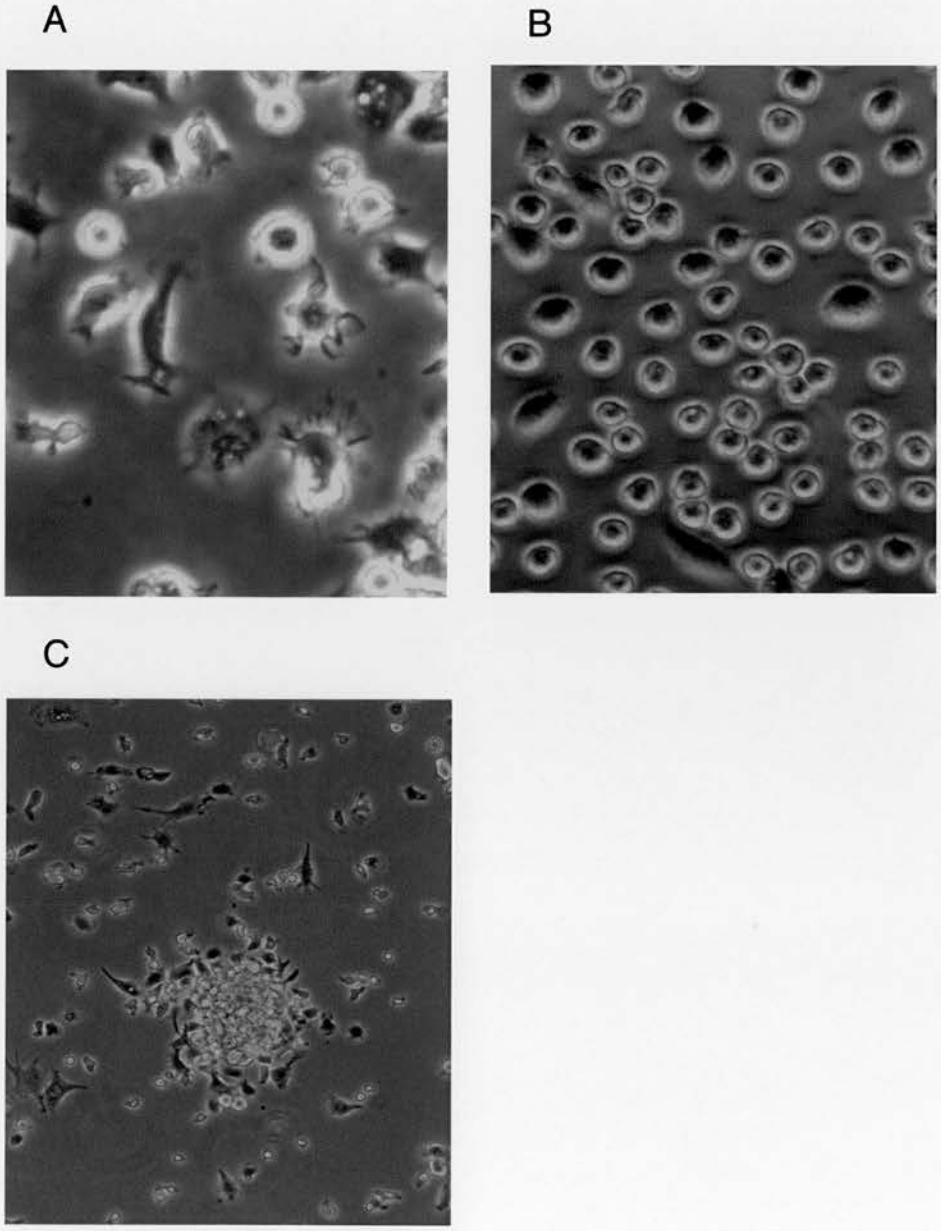
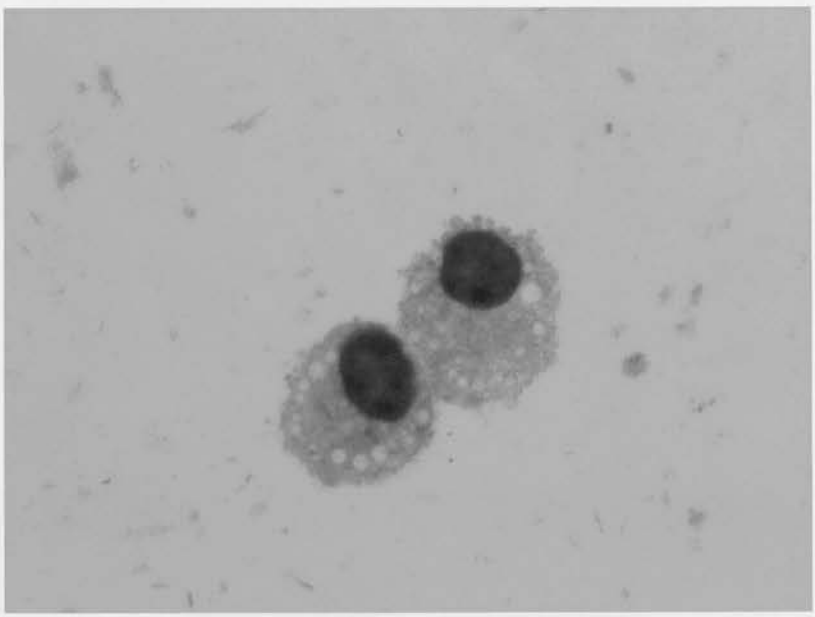


Figure 3.1: Phase contrast microscopy of macrophages and DCs. DCs (A) are heterogeneous, non adherent cells with multiple fine veils and dendrites observable in culture. Macrophages (B) demonstrate a strongly adherent monolayer population of homogeneous cells. Lower magnification (C) of DC cultures demonstrates typical loosely adherent 'grape-like' clusters.

A



B

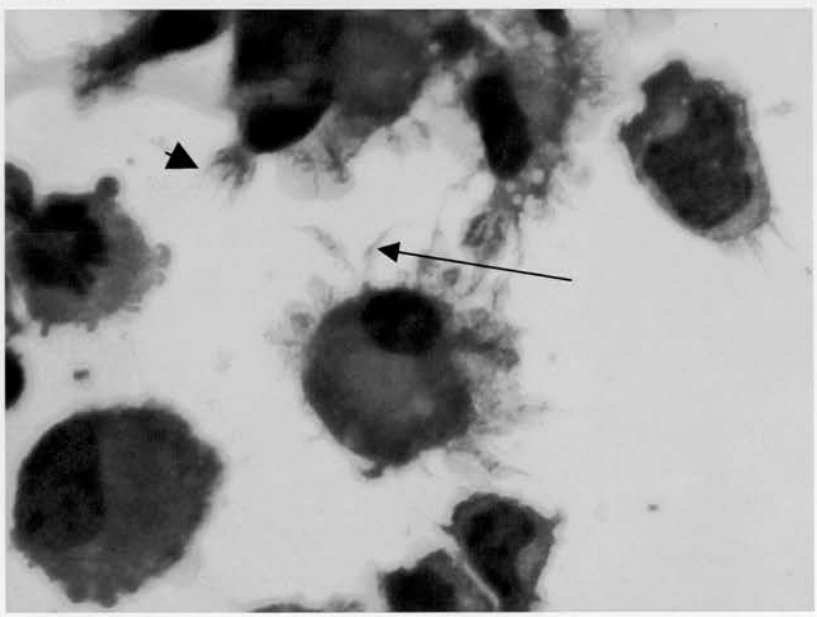


Figure 3.2: Cytospins of DCs and macrophages.
Macrophages(A) are more homogeneous with a vacuolated cytoplasm. In contrast, DCs (B) are heterogeneous and in a resting state have a more homogeneous cytoplasm. Morphology of the fine dendrites (arrow) and veils (arrowhead) is poorly maintained by cytospin but can be seen in some cells.

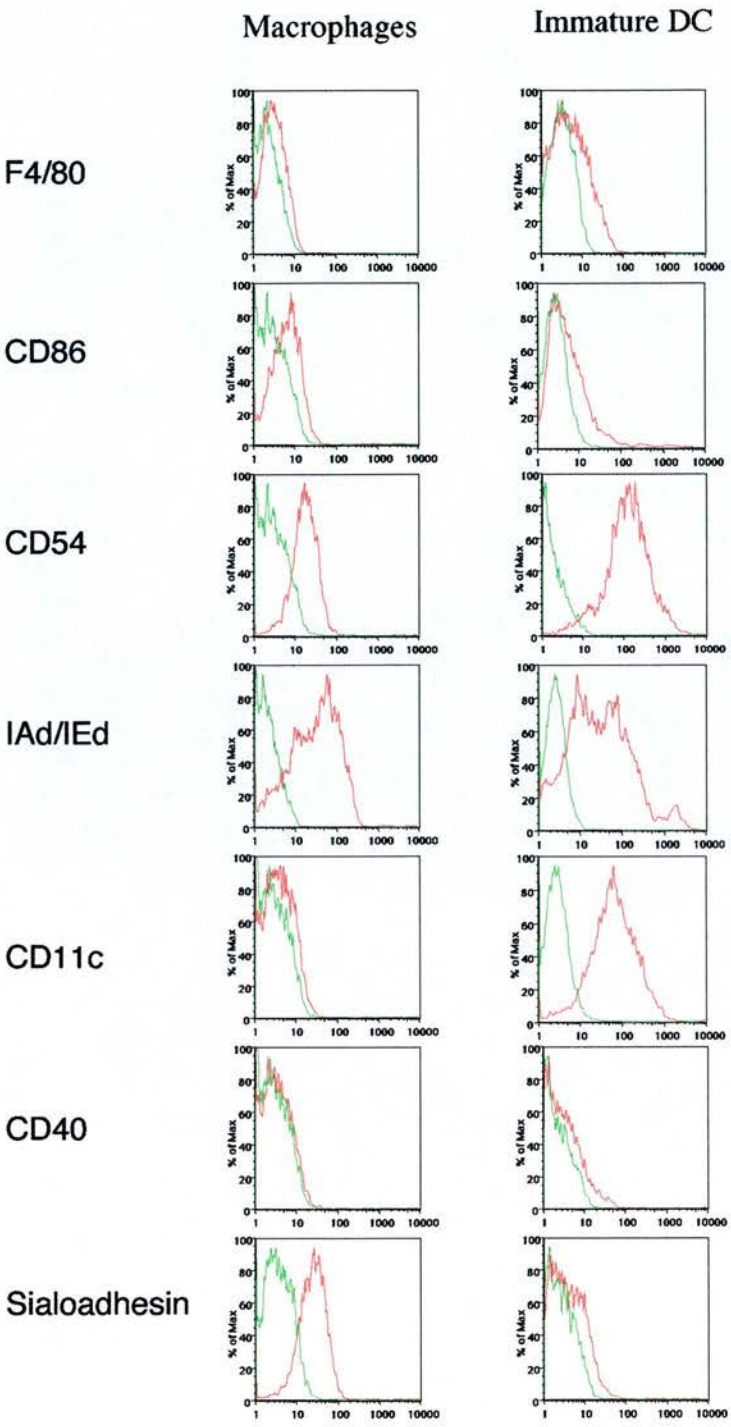


Figure 3.3: Surface phenotype of Macrophages and DCs.
Cultures of macrophages (7 days) or DCs (7 days) were harvested, stained for cell surface markers and analysed by FACS.

granulocytes (Fig 3.4), the percentage of which decreased over time with DC cultures being >90% pure by day 10. At day 7 these murine DCs were aheterogeneous population expressing varying levels of class II MHC and costimulatory molecules. In addition they expressed low levels of the macrophage marker F4/80, no CD14 and no sialoadhesin (both macrophage/monocyte specific) (Fig 3.3) (Fraser et al. 1994; Gordon 1999).

3.2.3 DC maturation

To determine whether these DCs were functional and responsive to maturation stimuli the ability of LPS and SAC to upregulate DC maturation markers was assessed (Winzler et al. 1997). Murine DC cultures were extremely heterogeneous for expression of a variety of costimulatory molecules. Costimulatory molecule CD86, was expressed at varying levels in resting cultures with most cells being low (or negative) although a few CD86 high cells could be found. The proportion of CD86 high DCs increased spontaneously during the culture period from day 7 to 10 and was also upregulated markedly upon stimulation with both LPS (increasing from 31 to 56%) and SAC (increasing from 31 to 71%) (Fig 3.5). CD86 expression in the whole population when expressed as geometric mean fluorescence intensity (GMFI) was found to be a reliable and objective marker of DC maturation in the bone marrow derived cells changing from low to high upon stimulation corresponding to aproximately 2.5 fold increase in CD86 GMFI when stimulated with LPS and 6 fold increase when stimulated with SAC (Fig 3.5) and was used for data analysis in most experiments. GMFI appeared to correlate well with percentage of DCs that were CD86 high and was used rather than mean fluorescence to prevent high expression of CD86 on some cells skewing results. Thus, using this marker, appropriate conditions required for DC stimulation were determined; maximal stimulation was achieved with smooth mutant E.Coli LPS at 0.5-1μg/ml (slight batch variations did exist) (Fig 3.6 and 3.7) and SAC at 0.02% vol (Fig 3.7) and doses in these ranges were used for all subsequent experiments. LPS has been reported to cause death of DCs but did not affect the viability and cell recovery

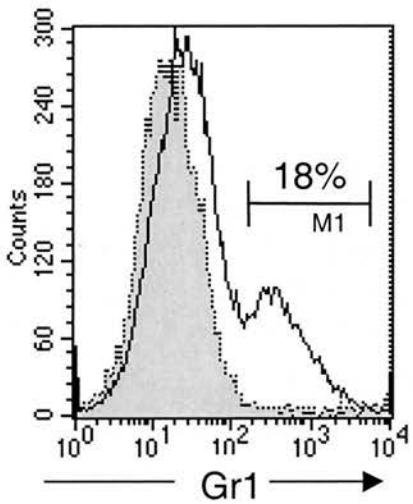


Figure 3.4: Identification of granulocytes as main contaminants in DC cultures. DC cultures at day 7 were stained for Gr-1 and analysed by FACS. Approximately 20 % of the cells were GR-1 positive, indicating granulocytes or cells of granulocyte lineage. The filled histogram represents cells stained with isotype control antibody.

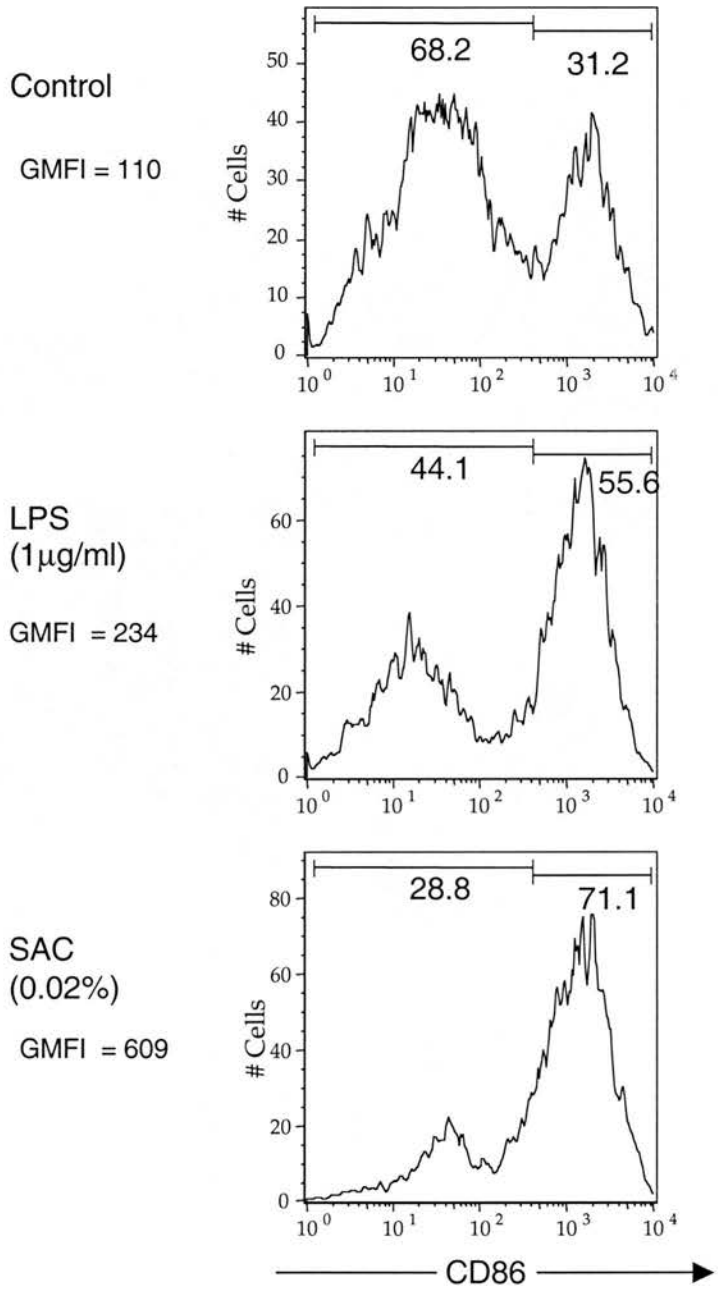


Figure 3.5: Response of DCs to maturation by bacterial products. DC cultures (day 7) were stimulated with LPS or SAC for 24 hours, harvested, stained for CD86 and analysed by FACS. Unlabelled cells and cells stained with isotype controls are in the first log order of fluorescence. CD86 GMFI and percentage CD86 high and low cells are shown.

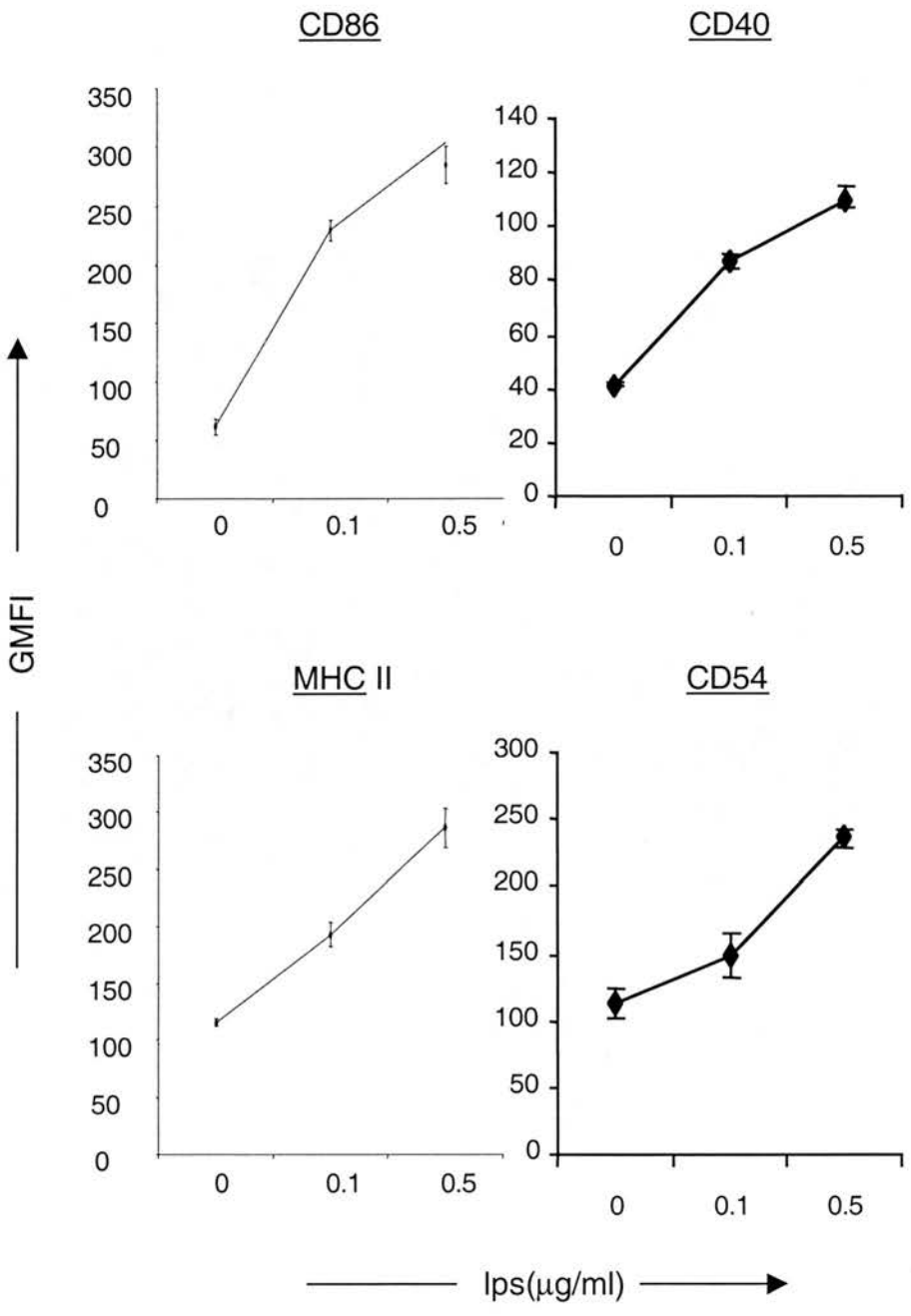


Figure 3.6: Dose response of DCs to maturation by LPS. DC cultures (day 7) were stimulated with LPS for 24 hours, harvested, stained for cell surface markers and analysed by FACS. Data are expressed as mean GMFI +/- s.d. representatitive of at least 3 independent cultures.

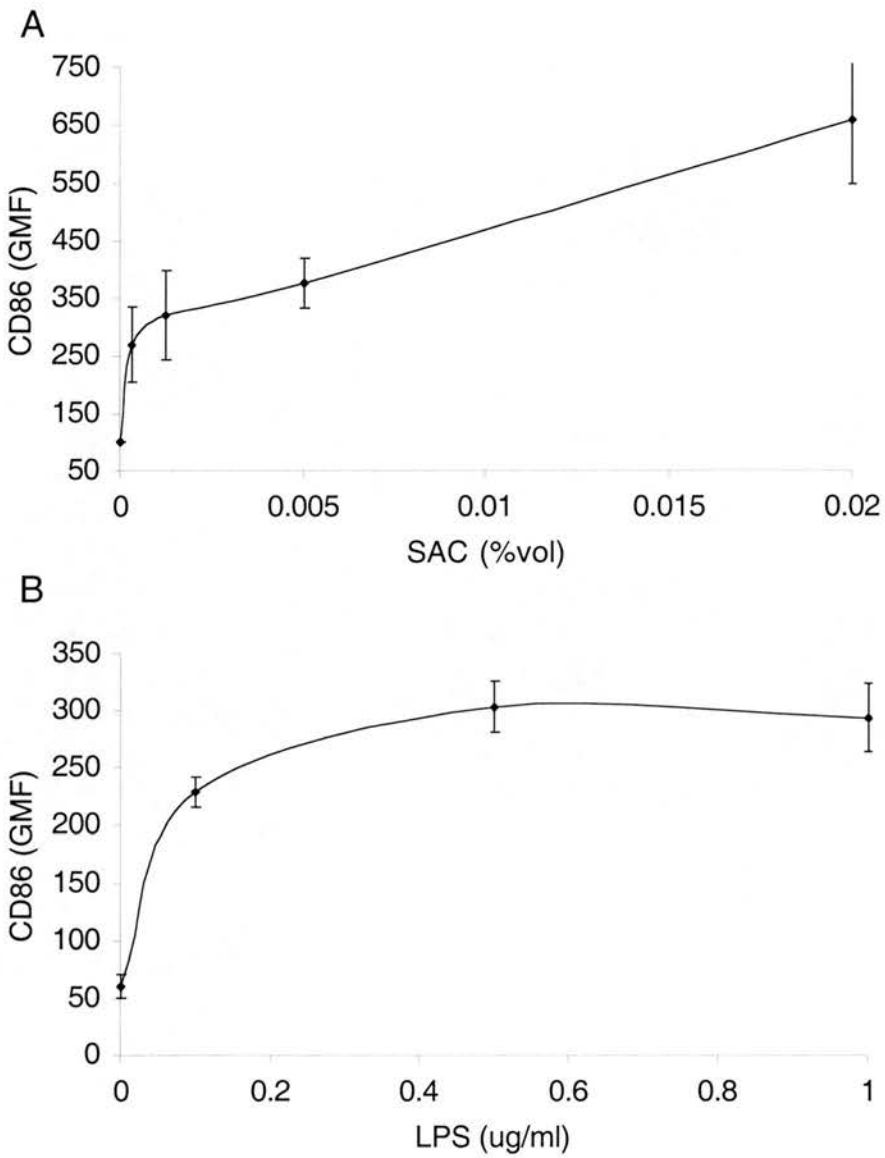


Figure 3.7: Dose response of DCs to maturation by microbial products. DC cultures (day 7) were stimulated with LPS or SAC for 24 hours, harvested, stained for cell surface markers and analysed by FACS. Data are expressed as mean GMFI +/- s.d. from 2 independent cultures.

over the 24 hours of culture. In some cultures a powerful stimulus to DC maturation is cluster disruption (Pierre et al. 1997). Interestingly, in our murine system, DC transfer on day 7 away from adherent contaminating macrophages by gentle washing, did not provoke extensive phenotypic changes or DC maturation after 24 hours unless additional stimuli were applied (Fig 3.8). Other costimulatory molecules were also assessed (Fig 3.9). Resting DCs expressed almost no CD40 but could be induced to upregulate it upon stimulation with LPS resulting in homogeneous expression of CD40 in most cells. CD80 was expressed in most resting DCs and was also upregulated upon stimulation in a manner similar to CD86 generating a mixed population of CD80 high and low cells. CD54 and MHCII also demonstrated staining patterns and response to LPS similar to CD86 with populations of both high and low expression in resting cultures and an increase in the percentage high cells upon stimulation.

3.2.4 Phagocytosis by murine bone marrow DCs

Initially fluorescent microscopy was used to confirm internalisation of apoptotic cells by dendritic cells. DCs were counterstained either with CD11c-PE and challenged with green fluorescent labelled apoptotic cells or stained fluorescent green with MHCII-FITC or green cell tracker dye (CMFDA) and cocultured with red fluorescent apoptotic cells. In both these situations DCs could be seen to efficiently internalise apoptotic cells/bodies (Fig 3.10).

It was then necessary to establish a flow cytometric-based phagocytosis assay to reliably study DC phagocytosis/endocytosis of fluorescent substrates. In most experiments DCs were gated either as CD11c positive cells or counterstained with fluorescent cell tracker dyes (Fig 3.11). The assays were performed in U-bottomed plates to maximise DC: dead cell contact with varying DC: apoptotic cell ratios. In a typical experiment (Fig 3.12), with apoptotic cell : DC ratios of 4:1, approximately 30 % of the DCs would internalise apoptotic cells, although significant culture to culture variation did exist. Furthermore, when the assay

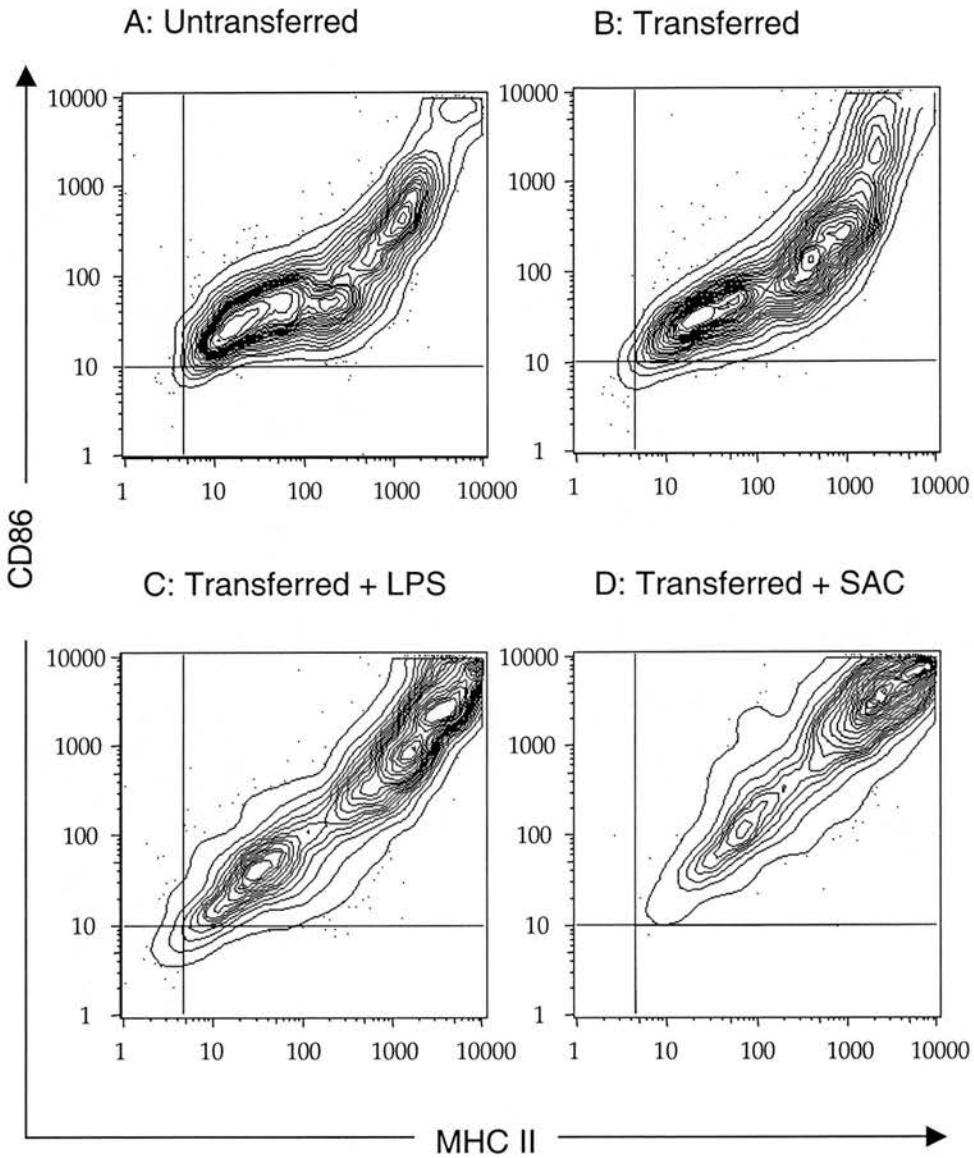


Figure 3.8: Maturation of DCs following transfer. DC cultures (day 7) were transferred to fresh culture plates without additional stimuli, or stimulated with LPS or SAC. 24 hours after transfer and stimulation, DCs were harvested and stained for cell surface markers and analysed by FACS. Figures show CD86 and MHC II staining on CD11c $^{+}$ cells. Marked gates show the upper limit of isotype antibody staining.

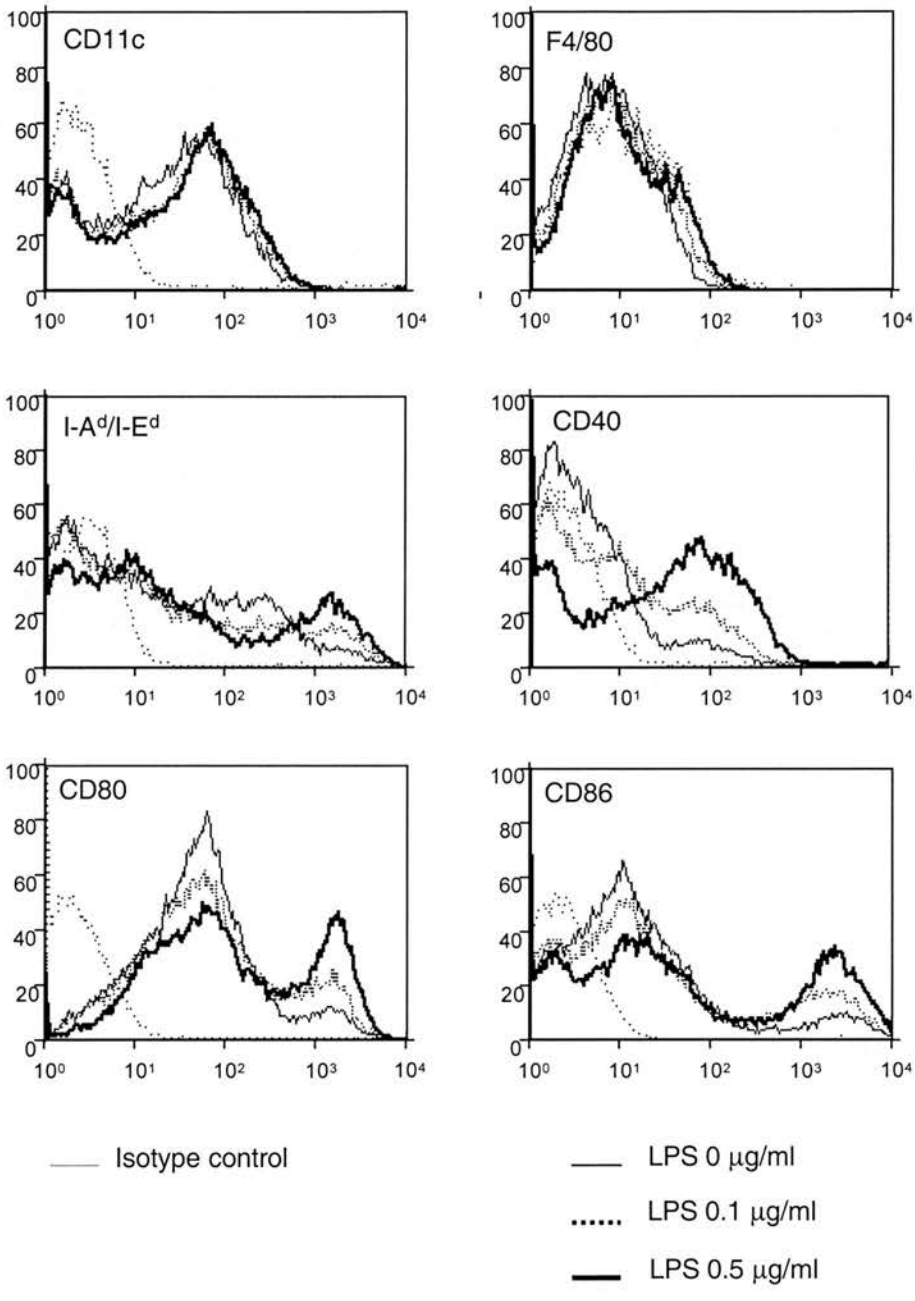


Figure 3.9: Maturation of DCs by LPS treatment. DC cultures (day 7) were stimulated with LPS. After 24 hours DCs were harvested, stained for cell surface markers and analysed by FACS. Figures show surface molecule staining on all cells.

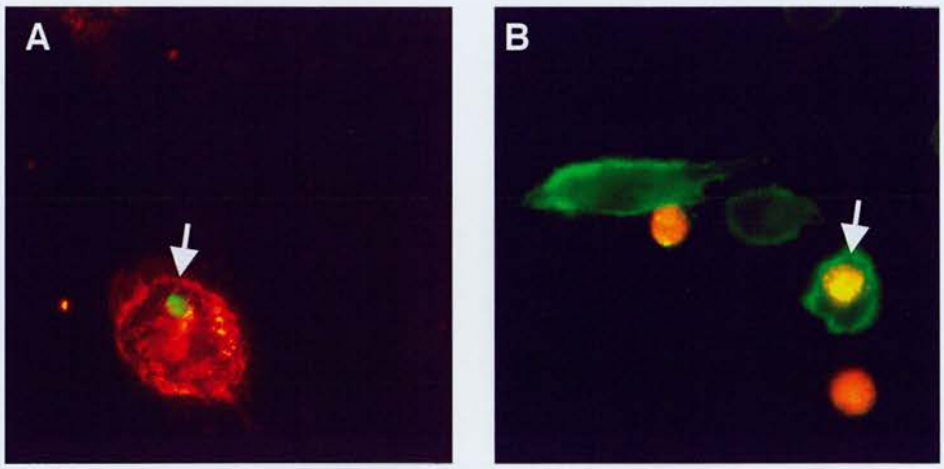


Figure 3.10: Phagocytosis by immature DCs (1). DC cultures (day 7) were incubated for 2 hours with apoptotic neutrophils labelled with green (A) or red (B) fluorescent tracker dyes, counterstained with CD11c-PE (A) or MHCII-FITC (B) and visualised by fluorescence microscopy under x 100 (oil) objective. Note internalised apoptotic cells (white arrow).

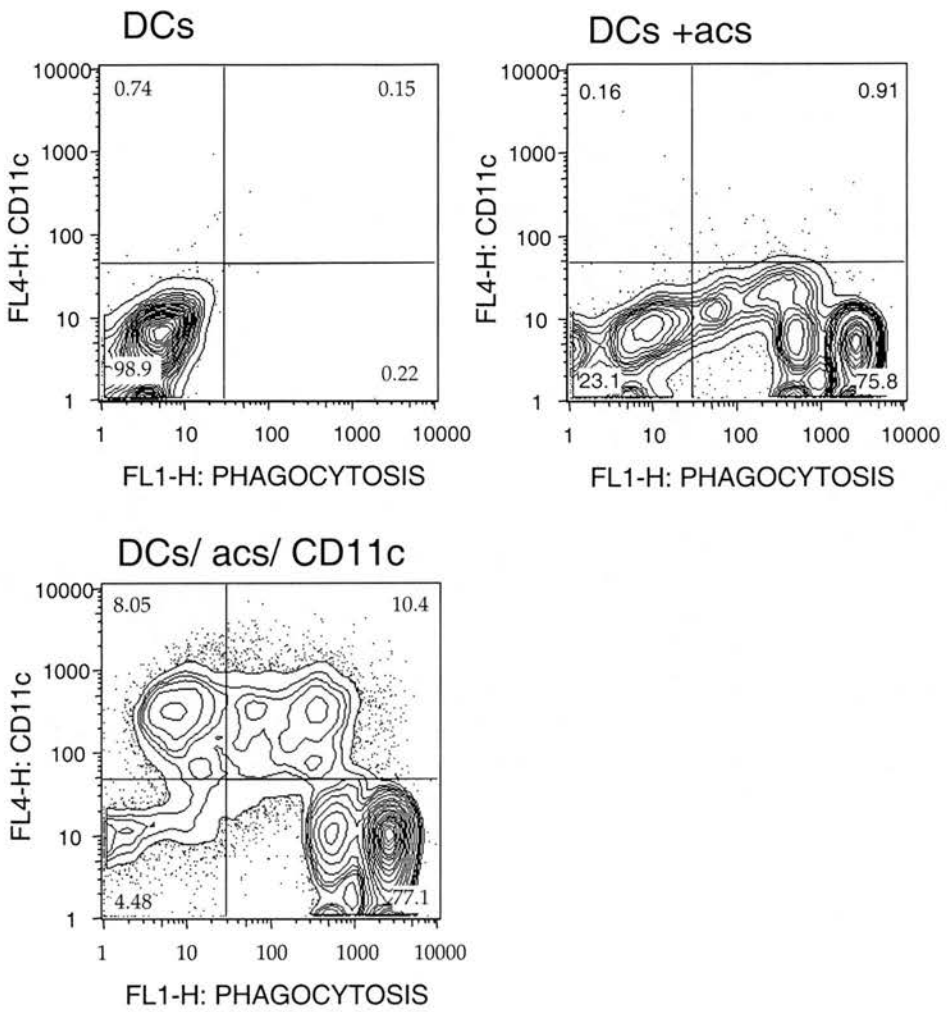


Figure 3.11: Apoptotic cell phagocytosis by DCs (1): Detection by FACS. DC cultures (day 7) were unstimulated or incubated with apoptotic neutrophils labelled with green fluorescent tracker dye, for 2 hours, counterstained with CD11c-APC and analysed by FACS. DCs can be clearly gated as CD11c positive and sub-divided into ac phagocytosing and non-phagocytosing cells. Numbers on FACs plots are percentage of cells in each quadrant region.

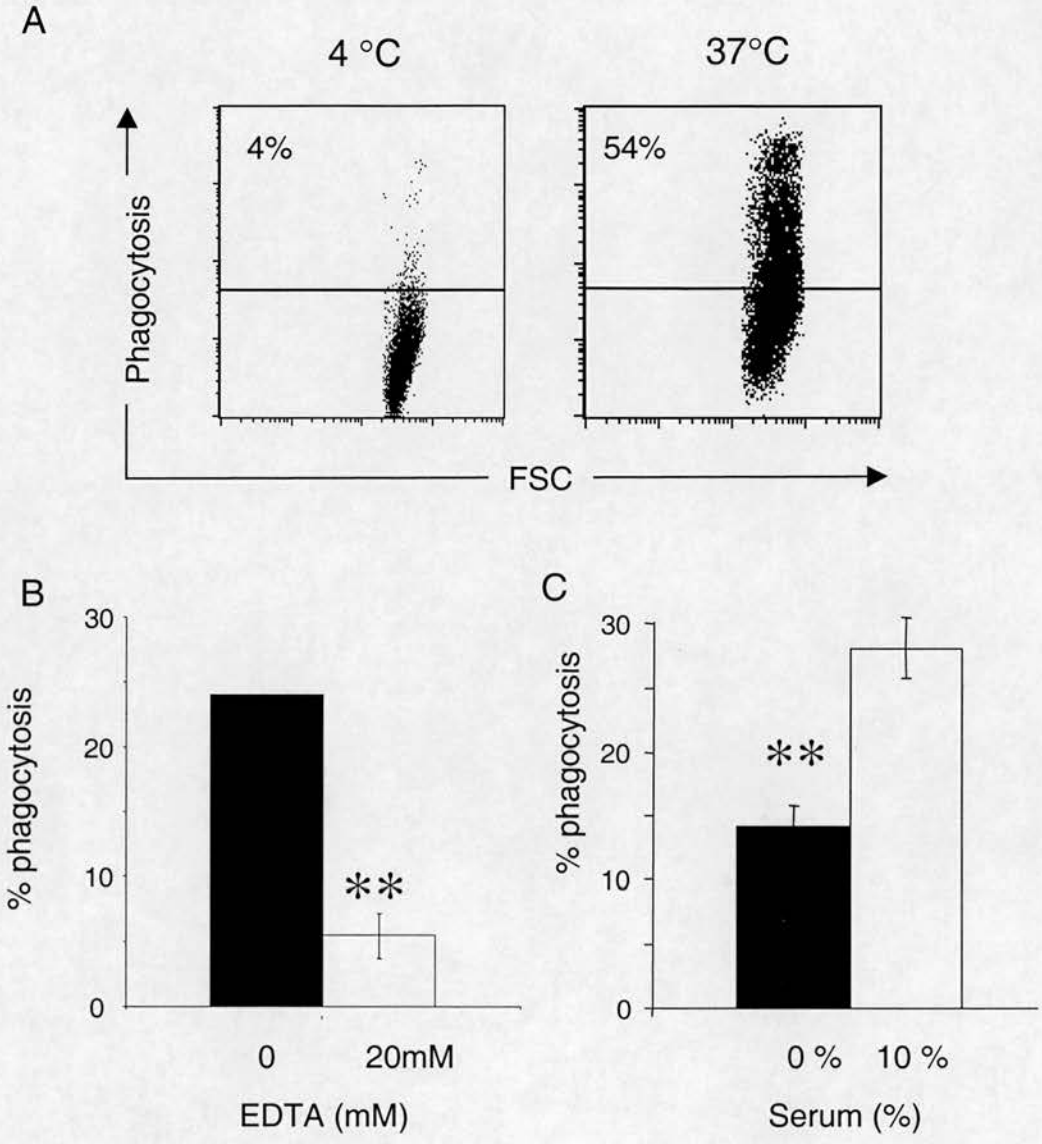


Figure 3.12: Apoptotic cell phagocytosis by DCs (2): Inhibition by temperature and EDTA and augmention by serum. DC cultures (day 7) were incubated for 2 hours with fluorescent green apoptotic neutrophils and phagocytosis measured by FACS. Phagocytosis was carried out at 4 °C or 37 °C (A), in the presence or absence of EDTA (B) or the presence or absence of serum (C). In B and C graphs are of mean +/- s.d. from triplicate wells representative of 3 independent experiments. ** represents significant difference from control ($p < 0.01$, ANOVA).

was performed in the presence of EDTA (Fig 3.12) phagocytosis was perturbed demonstrating that it did indeed detect specific DC:apoptotic cell interactions. To determine binding rather than internalisation the assay was also performed on ice and demonstrated only low levels of binding of DCs to apoptotic cells (typically 4-8% of DCs binding apoptotic cells) (Fig 3.12). Furthermore, performing the assay in the presence or absence of serum allowed determination of the contribution of serum components (Fig 3.12), which approximately doubled the proportion of DCs ingesting apoptotic cells. A dose response of apoptotic cells demonstrated that the efficiency of DC internalisation of apoptotic cells increased with increasing number of dying cells (Fig 3.13) but never reached the level of efficiency demonstrated for internalisation of control particles such as latex beads. This assay was also used to determine the kinetics of the interaction; phagocytosis increased with time, peaking at 2 hours (Fig 3.13) after which there was a slight loss of fluorescence within the ac+DCs perhaps due to redistribution/'digestion' of internalised dye. Despite this loss of fluorescence, the cell-tracker dye could still be detected within the phagocyte up to 24 hours after the interaction both by microscopy and flowcytometry and hence it was possible to use this assay to follow the fate of DCs that had internalised apoptotic cells (ac+DCs) at a single cell level over this period of time.

To confirm that this assay was detecting internalised apoptotic cell or apoptotic cellular fragments, ac+DCs and ac-DCs were sorted by flow cytometry. ac+DCs contained either whole apoptotic cells, apoptotic bodies or apoptotic cell derived fluorescence whilst ac-DCs had no evidence of ac associated fluorescence (Fig 3.14). It was unlikely that ac associated fluorescence was derived merely from internalisation of debris or free dye as deliberate cell lysis (by multiple freeze thaw or hypotonic treatment) destroyed ac associated fluorescence which could not then be detected in the fluorescent assay. Thus it was likely that any apoptotic cell-associated fluorescence represented internalised and 'digested' particles.

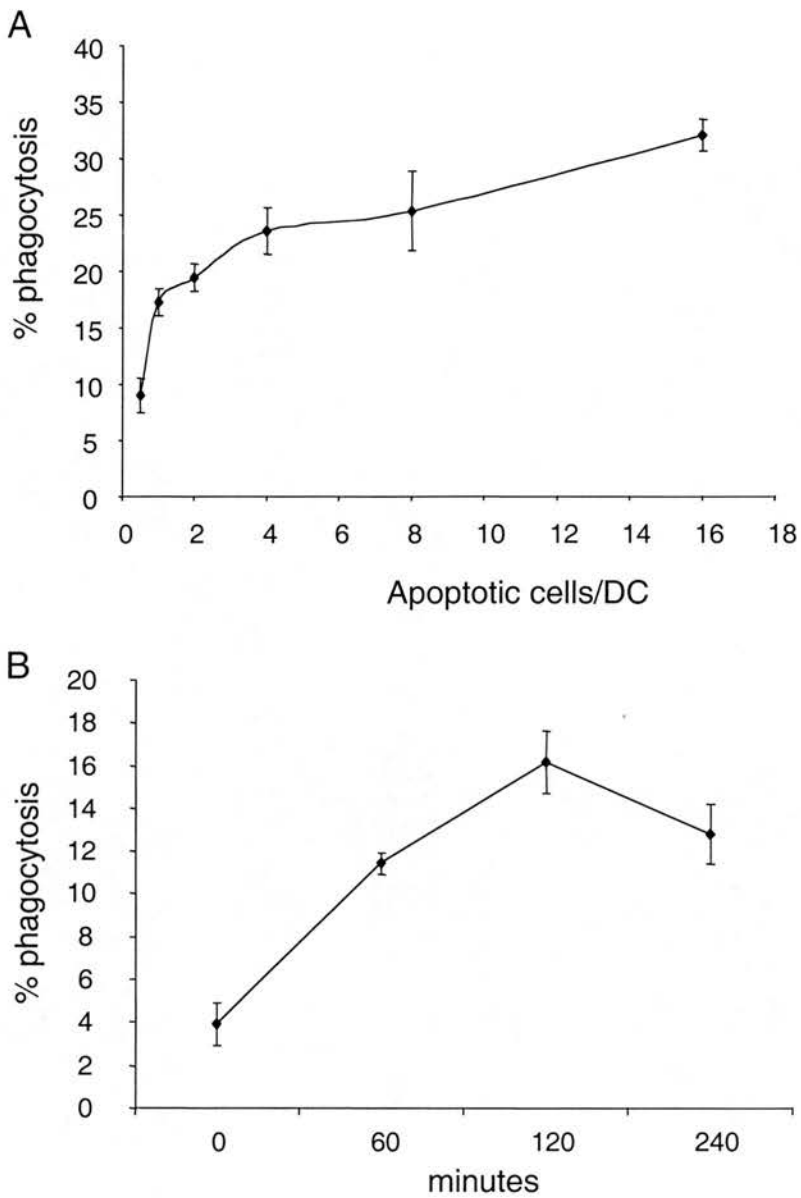


Figure 3.13: Apoptotic cell phagocytosis by DCs (3): Dependence on DC/ ac ratio and time. DC cultures (day 7) were incubated with fluorescent green apoptotic neutrophils and phagocytosis measured by FACS. **A:** Phagocytosis was carried out for 2 hours with a constant number of DCs ($0.2x10^6$ cells in 0.2 ml) and increasing numbers of apoptotic cells ($0.1\text{-}3.2x10^6$). **B:** Phagocytosis by $0.2x10^6$ cells DCs with $0.4x10^6$ cells apoptotic cells was stopped after 60 - 240 minutes by placing on ice. 0 time controls are from DCs and apoptotic cells mixed on ice. Data are expressed as mean +/- s.d. from triplicate wells, representative of 3 independent experiments.

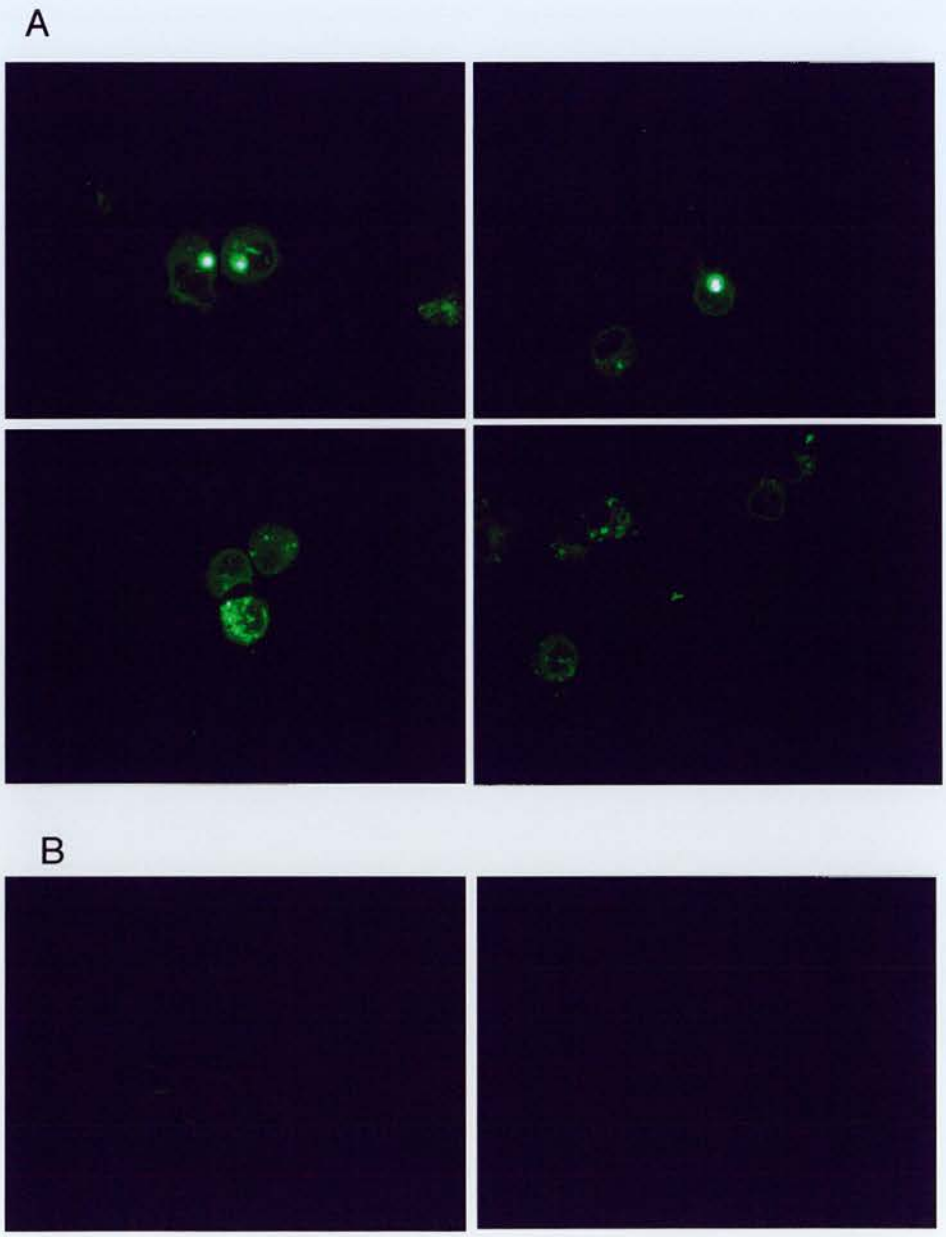


Figure 3.14: Apoptotic cell phagocytosis by DCs (4): Confirmation of FACS assay. DC cultures (day 7) were incubated with fluorescent green apoptotic neutrophils for 24 hours, and sorted. DCs were identified by size, and separated into DCs with (A) and without (B) associated green fluorescence. Pictures are of cytospins of sorted cells visualised by fluorescence microscopy (x 100 (oil) objective). Panels in B are exposed for the same time as (A), and contain DCs when visualised by bright field microscopy (not shown)

3.2.5 Morphology after phagocytosis

DCs are heterogeneous cells *in vivo* and *in vitro*. It was noted that after phagocytosis, DCs became larger and more granular. Cytospin preparations of DCs after coculture with apoptotic cells demonstrated certain interesting characteristics; DCs developed cytoplasmic protrusions and intracellular inclusions and an asymmetric nucleus (Fig 3.15). EM examination confirmed internalisation of apoptotic cells, found typically within tight phagosomes (Fig 3.16).

3.2.6 Endocytosis and phagocytosis of control particles by immature DCs

DCs also internalise antigen by both receptor mediated and non-receptor mediated endocytosis and phagocytosis (Austyn 1996). Firstly I wished to demonstrate the phagocytic capacity of immature DCs and addition of 2μm fluorescent latex beads to DC cultures demonstrated that they phagocytosed these inert particles with high efficiency (>80%). Internalisation of oxidised lipid represents an interesting example of receptor-mediated endocytosis as it shares with apoptotic cells a common recptor, CD36 (Febbraio et al. 2001). Although the exact component of the apoptotic cell that is recognised by CD36 is unknown, it is likely that it recognises altered lipid components in the cell wall of dying cells. It is controversial whether murine CD36 binds oxidised lipid but it was considered an ideal control to study the effect of receptor-mediated endocytosis on DCs. Using labelled substrates it was demontrated that immature day 7 DCs can effectively internalise FITC-dextran (Fig 3.17) and lipid (Fig 3.18), both of which could be visualised by fluorescent microscopy (Fig 3.19). Interestingly, although all immature DCs internalised oxidised lipid, a subpopulation performed this task with much higher efficiency (Fig 3.18).

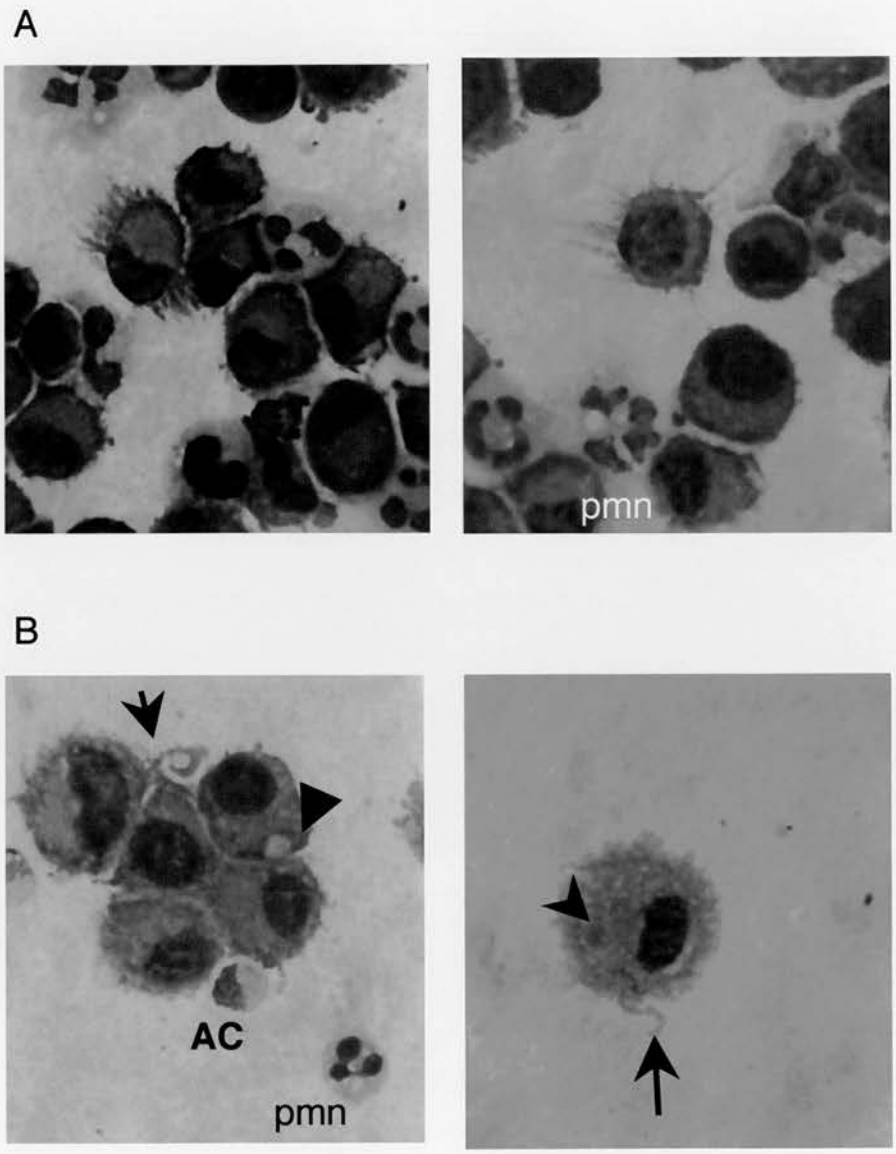


Figure 3.15: Apoptotic cell phagocytosis by DCs (5): Morphology of phagocytosing cells. Cytospins were prepared from unstimulated DCs (A) or DCs incubated with apoptotic cells (B), and viewed under x 100 (oil) objective. **A**: unstimulated DCS show finer dendrites and a homogenous cytoplasm. **B**: DCs co-cultured with apoptotic cells are larger and demonstrate a distinct morphology with blunt pseudopodia (arrow), granular inclusions (arrowhead)and an asymmetric nucleus. Note contaminating live murine granulocytes in these cultures (pmn). AC=apoptotic cell

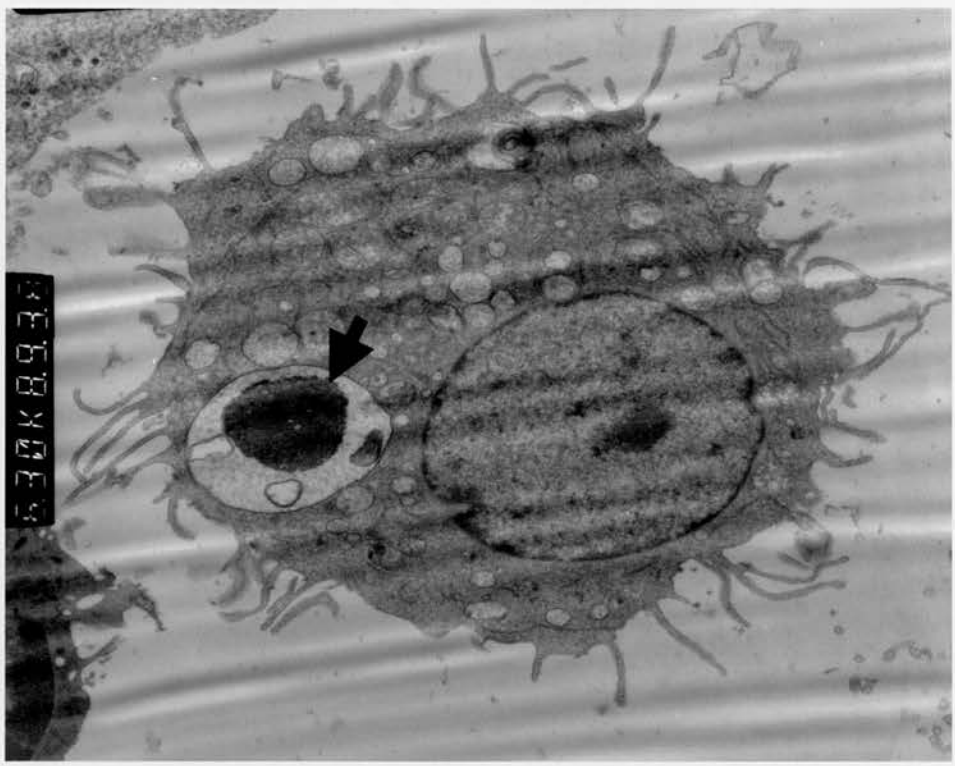


Figure 3.16: Apoptotic cell phagocytosis by DCs : Confirmation by transmission electron microscopy (TEM). DCs incubated with apoptotic cells were fixed and prepared for TEM. An apoptotic body can be clearly seen inside the cytoplasm of a DC (Arrow). TEM was viewed at x 10,000 magnification.

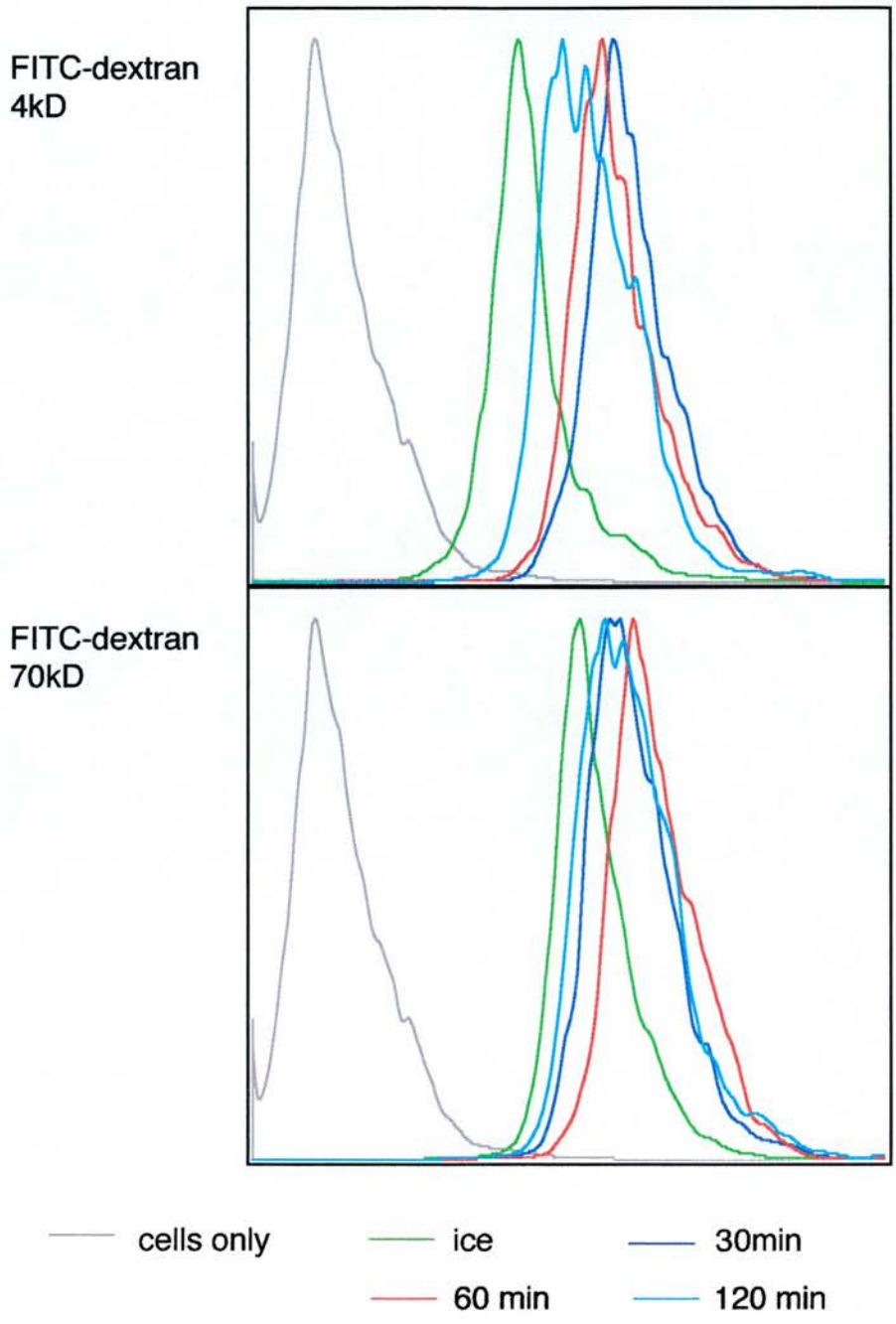


Figure 3.17: Endocytosis by immature DCS (1): dextran. DCs (day 7) were incubated with 1 mg/ml 4 kDa or 70 kDa FITC-dextran. After 0 - 120 minutes cells were quenched and endocytosis measured by incorporation of FITC into DCs, using FACS. Histograms are green fluorescence associated with DCs, gated by size.

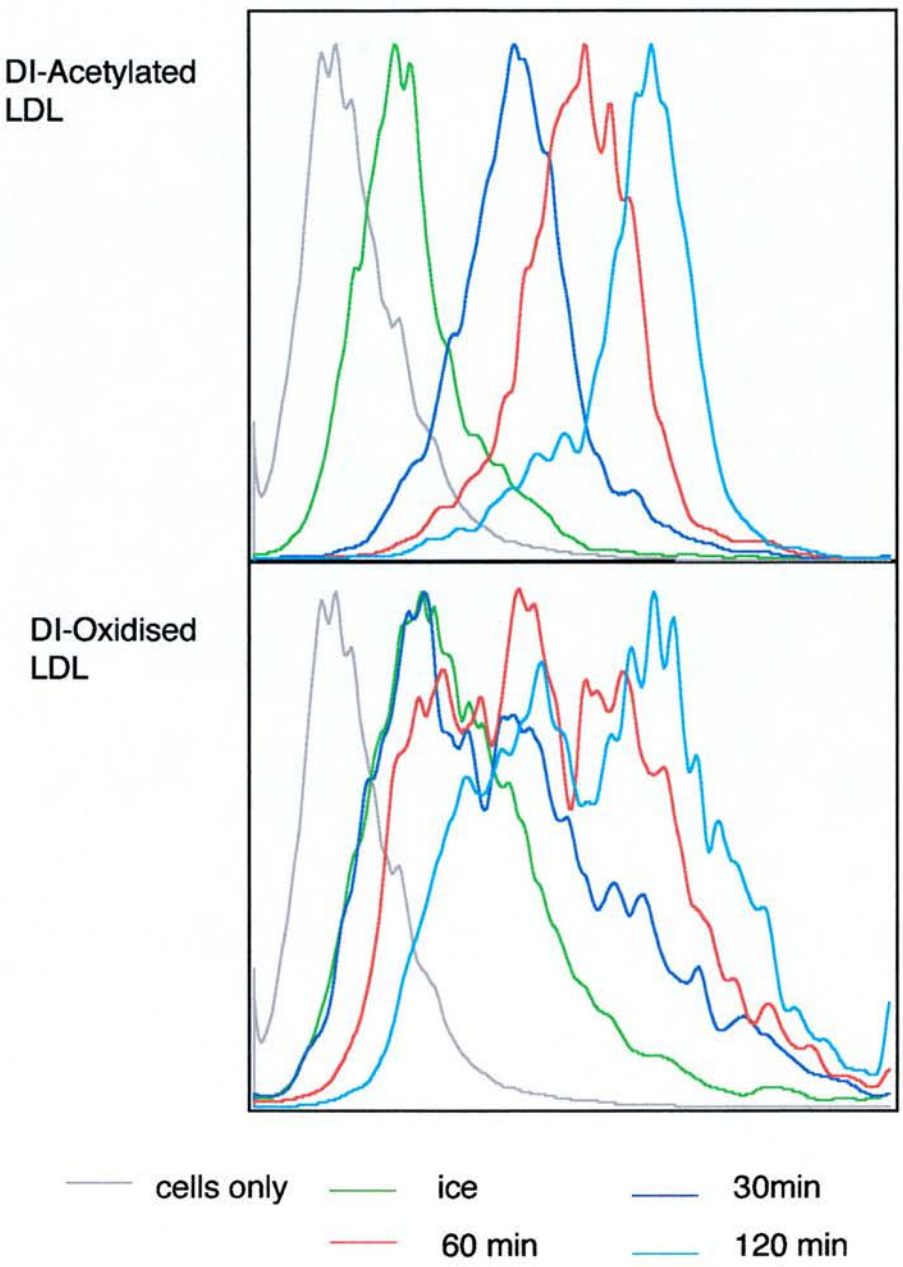
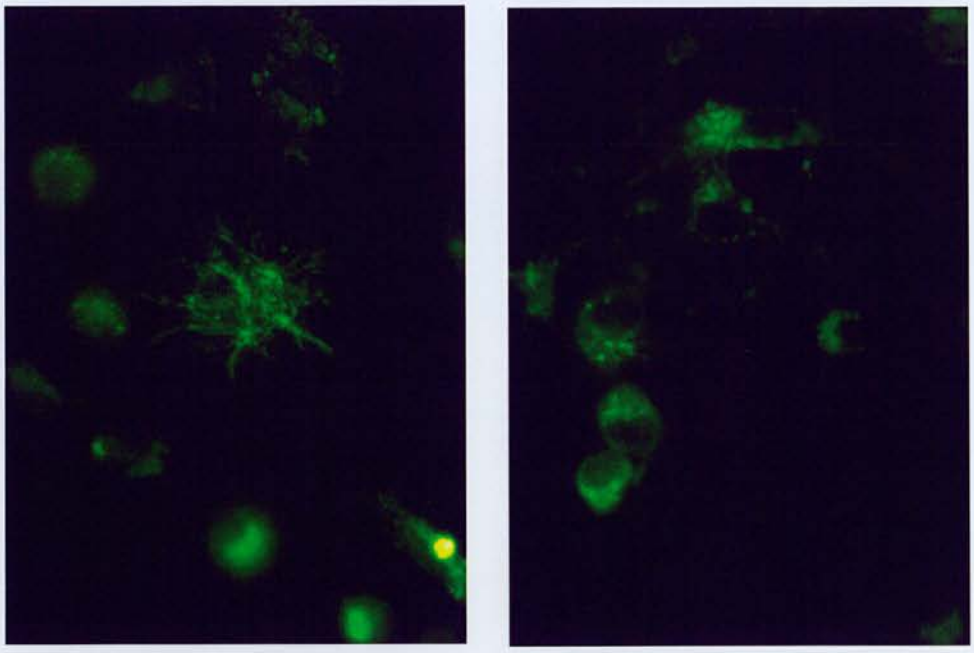


Figure 3.18: Endocytosis by immature DCS (2): LDL. DCs (day 7) were incubated with 10 μg/ml Ii-labelled acetylated or oxidised LDL. After 0 - 120 minutes cells were fixed and endocytosis measured by incorporation of DI into DCs, using FACS. Histograms are red fluorescence associated with DCS, gated by size. Note a biphasic peak of oxLDL positive cells suggesting either degeneration of oxLDL or heterogenicity of receptor expression .

A



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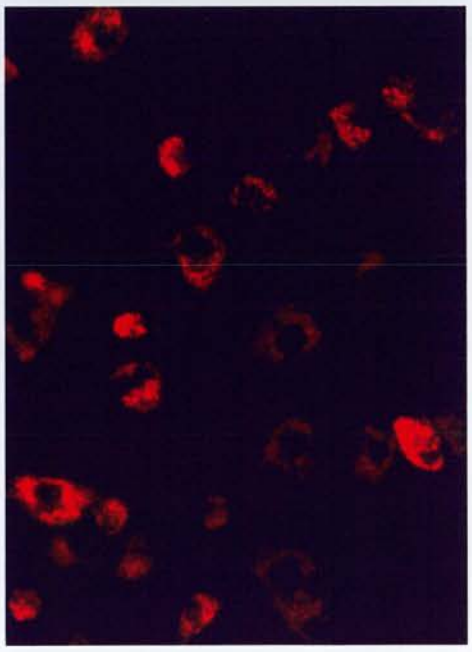


Figure 3.19: Endocytosis by immature DCS (3): Dextran and LDL. DCs (day 7) were incubated with 1 mg/ml FITC-dextran (A) or 10 μg/ml DI-labelled oxidised LDL (B). After 240 minutes cells were fixed and examined by microscopy. DI-OXLDL is concentrated in vacuoles demonstrating scavenger receptor dependant endocytosis.

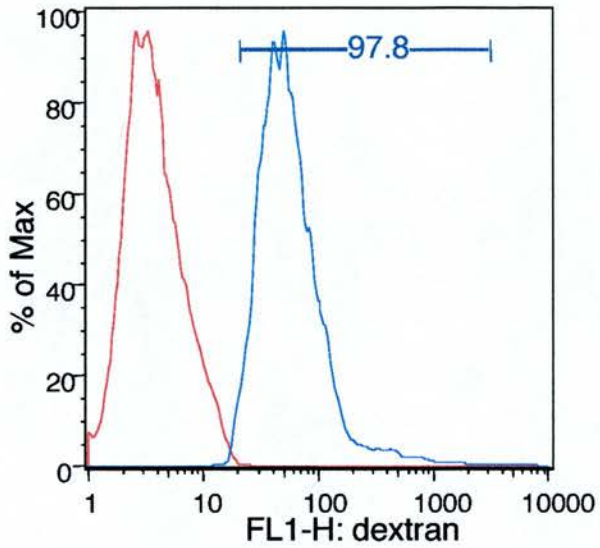
Furthermore, by pulsing DCs with apoptotic cells and FITC-dextran it was apparent that that the requirements for phagocytosis of apoptotic cells were more stringent than endocytosis and that only 25% of DCs able to endocytose actually internalised apoptotic cells (Fig 3.20). It has been shown previously that DCs that have internalised dextran are able to export it into the cytosol via an endosomal-cytosolic pore able to transport substances less than 40kD. The cell tracker dye used for these experiments becomes covalently linked to intracellular proteins and hence allowed us to track the fate of internalised apoptotic cell-associated proteins. Interestingly, in these experiments, using different sizes of dextran it could be demonstrated that dextran of 4kD 'leaked' from the endosomal compartment into the cytoplasm as described previously (Rodriguez et al. 1999b). However, no such movement was demonstrable for apoptotic cell associated fluorescence or 70kD dextran, which remained mostly co-localised in endosomal compartments at 6 hours (Fig 3.21).

3.3 Discussion

Here I demonstrate that murine bone marrow DCs, like human monocyte derived DCs, demonstrate many of the characteristics attributed to DCs *in vivo*. Firstly, these cells express the cell surface markers and morphological characteristics of DCs. In addition, their maturation in response to stimulus by bacterial products could readily be demonstrated as assessed by upregulation of costimulatory molecule and class II MHC expression.

These murine bone marrow DCs were able to internalise antigen by endocytosis and whole cells by phagocytosis as demonstrated in our fluorescent phagocytosis assay. In confirmation of this, apoptotic cells or control endocytic particles were visualised within DCs by fluorescent microscopy. The requirements for apoptotic cell phagocytosis were presumably somewhat stringent as not all of the immature endocytic DCs would internalise apoptotic

A FITC-dextran uptake



B Apoptotic cell uptake

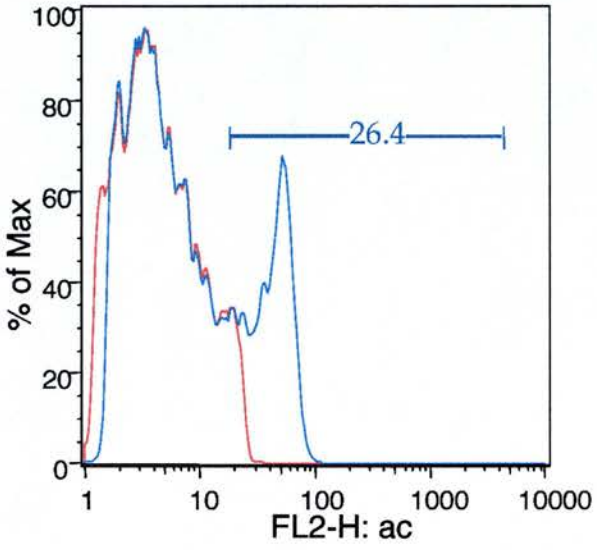
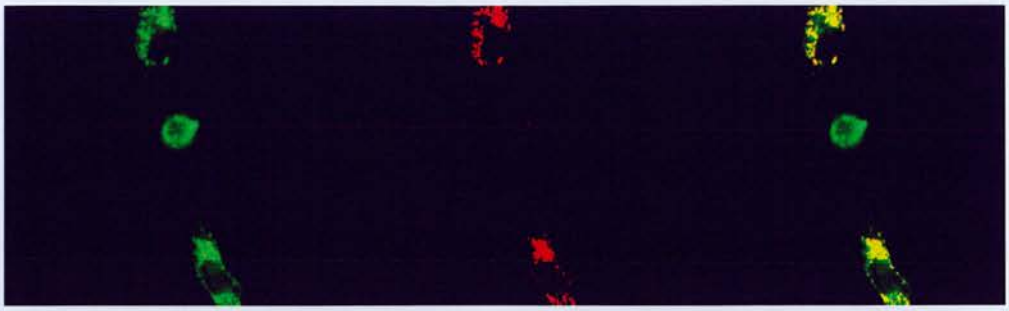
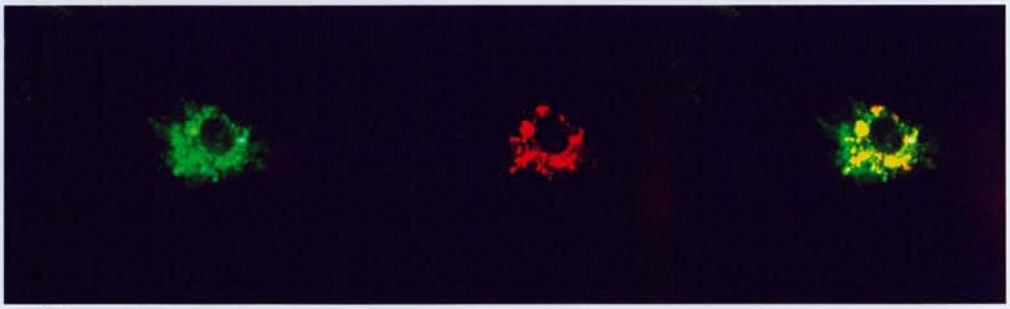


Figure 3.20: Comparison of endocytosis and phagocytosis by DCs. DCs (day 7) were co-challenged with both FITC-dextran and fluorescent red apoptotic cells for 2 hours and analysed by FACS. DCs were gated by size and green and red fluorescence measured. Histograms show both control DCS (red lines) and DCs incubated with fluorescent dextran/ acs (blue lines). Numbers show percentage of cells that are more fluorescent than control cells. Although the majority (98%) of immature DCs internalised dextran only a small percentage (26%) phagocytosed apoptotic cells.

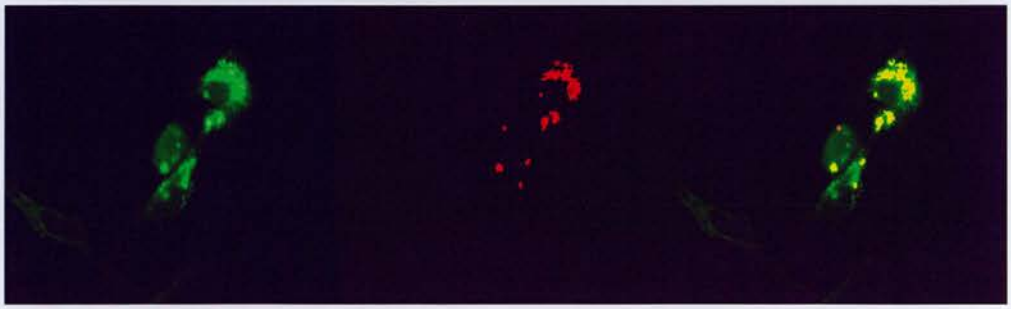
A: 70kD FITC-Dextran, 1hour



B: 70kD FITC-Dextran, 6hours



C: 4kD FITC-Dextran, 1hour



D: 4kD FITC-Dextran, 6hours

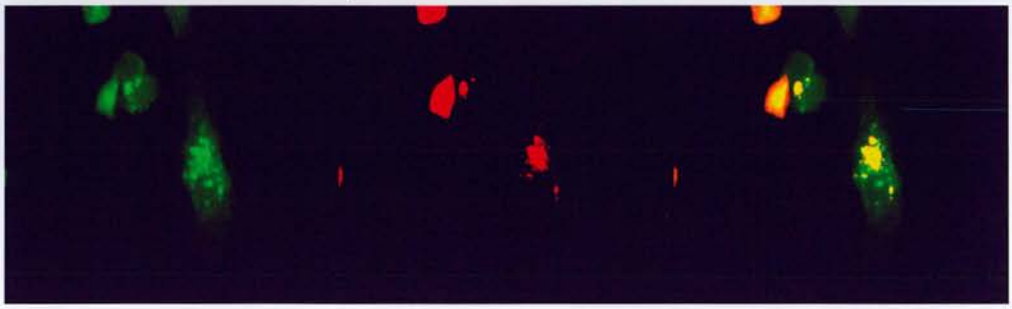


Figure 3.21: The fate of the internalised material. DCs pulsed with 70kD (A and B) and 4kD (C and D) FITC-dextran and red fluorescent apoptotic cells were examined 1 hour (A and C) and 6 hours (B and D) after the interaction to track the fate of the internalised material. Panels show green (dextran), red (apoptotic cells) and green/ red overlayed images. Apoptotic cell associated fluorescence co-localised with 70kD dextran at 1 and 6 hours whilst by 6 hours 4kD dextran had 'leaked' from the endosomal pathway into the cytosol.

cells when co-challenged. This implied that DC phagocytosis of apoptotic cells required not only an immature DC but additional machinery, such as cell surface receptors or actin polymerisation, to confer phagocytic capacity. The exact nature of that machinery will be investigated later in this thesis.

In addition, the morphology of DCs challenged with apoptotic cells was subtly changed; DCs increased in size, demonstrating granular cytoplasmic inclusions and an asymmetric nucleus along with blunt cytoplasmic protrusions. These changes mirror many of the changes described in the OX41-/CD4- rat DCs (Liu, L. et al. 1998), recently found to contain apoptotic inclusions *in vivo* (Huang, F. P. et al. 2000). We postulate that the phenotypic differences between these and their CD4+/OX41+ counterparts may not represent a distinct subset of DCs but rather the consequences of internalisation of apoptotic cells and can be modelled *in vitro* by challenging DCs with apoptotic cells in culture.

The phagocytosis of apoptotic cells has been shown to be a preferential source of antigen for cross-priming (Bellone et al. 1997; Albert et al. 1998b; Inaba et al. 1998). The exact mechanism by which apoptotic derived antigen accesses the class I pathway is not fully understood, but one postulated mechanism is via a DC specific endosomal pore which allows 'leak' of endosomal proteins into the cytosol where they can access the endogenous pathway for antigen presentation (Rodriguez et al. 1999b). In contrast to 4kD dextran, which 'leaks' into the cytoplasmic compartment , ac associated fluorescence is found co-localised with 70kD dextran. Thus ac-associated proteins appear to remain within the endosomal compartment and, although we must interpret these initial observations with caution, it is possible that apoptotic cell derived antigen utilise another route to access the endogenous pathway. Instead I favour the possibility that apoptotic-derived antigen is processed and loaded onto MHC in the endosomal compartment or chaperoned into the ER by calreticulin or heat shock proteins (HSP), implicated along with CD91 in apoptotic cell phagocytosis in macrophages (Basu et al. 2001; Ogden et al. 2001; Srivastava 2002a).

CHAPTER 4: INHIBITORY EFFECTS OF DYING CELLS UPON DENDRITIC CELL MATURATION AND FUNCTION

4.1 Introduction

As was shown in the previous chapter, immature DCs (akin to those that exist in the periphery) capture and process exogenous antigen. Upon receipt of maturation stimuli DCs migrate to draining lymph nodes, a process associated with phenotypic changes, including down regulation of their antigen capturing machinery, upregulation of MHC and costimulatory molecules and production of IL-12, becoming fully functional antigen presenting cells. This maturation process is affected by a variety of endogenous or exogenous factors and can be modeled *in vitro* by stimulation with LPS and other microbial products.

Ingestion of bacteria (and other pathogens) signalling via a variety of Toll-like receptors or certain necrotic cells (immortalised/tumour cells) is capable of inducing DC maturation (Sauter et al. 2000). The exact components of necrotic cells able to activate DCs are not fully defined but one possibility is production of heat shock proteins (Basu et al. 2000; Srivastava 2002b) and other mediators also capable of inducing DC maturation by Toll-like receptors. In contrast, ingestion of apoptotic cells fails to activate DCs, appearing to be an immunologically null event (Gallucci et al. 1999; Sauter et al. 2000). However, apoptotic cell ingesting DCs have been shown, after concurrent maturation with the strong external stimulus of monocyte-conditioned media, to mature and present antigen derived from the ingested apoptotic cells to T cells (Albert et al. 1998b).

A growing body of evidence implicates DCs that ingest dying cells in influencing self-tolerance. One role of DCs is to constantly sample peripheral self-antigens and present them in a 'tolerogenic' way to the adaptive immune system (Kurts et al. 1997b; Hawiger et al. 2001). Another is the clearly demonstrated ability of DCs to cross-present cell-derived antigen, including those originating from apoptotic cells, to induce a T cell response (Albert et al. 1998b). Thus a dichotomy exists in the role of DCs which may be either 'friend

or foe' in maintaining tolerance. The ability of a DC to deliver 'signal two', either as costimulation or IL-12, singly or in combination, appears key in determining subsequent immune responses and is likely to be tightly controlled. Interestingly the ingestion of apoptotic cells by macrophages generates an active anti-inflammatory response through the production of TGF-β1 and other anti-inflammatory molecules (Voll et al. 1997b; Fadok et al. 1998b; Fadok et al. 2000), and down regulates subsequent release of pro-inflammatory cytokines. Because of the close lineage relationship of macrophages and myeloid DCs, we chose to investigate whether apoptotic cell ingestion by DCs might not only fail to activate DCs but might actively inhibit their maturation with particular regard to costimulatory molecule expression and IL12 production.

This chapter demonstrates that immature murine bone marrow-derived DCs ingesting apoptotic cells become functionally distinct. We confirm that apoptotic cells do not mature DCs but demonstrate that endotoxin-induced production of IL12, but not TNFα is selectively diminished in DCs that have ingested apoptotic cells. In addition, endotoxin driven upregulation of the co-stimulatory molecule CD86 is inhibited in those DCs that had phagocytosed apoptotic cells, but not in neighbouring DCs. The functional consequences of these changes are demonstrated as these DCs have a reduced capacity to stimulate T cell proliferation. Furthermore, many of these anti-inflammatory effects can be seen after DCs internalize cells that have been rendered 'necrotic' by heat, a process associated with externalization of phosphatidlyserine and loss of membrane integrity demonstrating that 'necrotic' cell death is heterogeneous and can result in non-phlogistic clearance and induction of an immunosuppressive response.

4.2 Results

4.2.1 Ingestion of apoptotic cells specifically inhibits the ability of DCs to upregulate CD86

To ascertain whether ingestion of apoptotic cells alters DC phenotype, cell surface expression of activation markers was studied by flow cytometry after phagocytosis. Although phagocytic DCs expressed slightly lower levels of CD86 as would be expected in cultures with some spontaneous maturation, no significant difference in cell surface expression of costimulatory molecules was seen between immature DCs that had ingested apoptotic cells (ac^+DCs) and those that had not (ac^-DCs) immediately (2 hours) (Fig4.1) after phagocytosis confirming that ingestion of apoptotic cells did not activate DCs. Similarly, 24 hours after ingestion unstimulated ac^+ and ac^- DCs also had comparable levels of CD40, CD86 and CD54 (Fig4.2). However, after 24 hous of DC maturation with LPS a marked difference in surface expression of CD86 was detected between ac^+ DCs and ac^- DCs (Fig4.2). Immature DCs were predominantly $CD86^{lo}$ with a small population of $CD86^{hi}$ cells. Upon maturation driven by LPS the proportion of $CD86^{hi}$ cells increased in a dose dependent manner. However, fewer ac^+ DCs became $CD86^{hi}$ compared to ac^- DCs; in a typical experiment at 0.1 μg/ml LPS only 13.2% of ac^+DCs became $CD86^{hi}$ vs 42% ac^-DCs, and this difference was maintained at the highest LPS dose of 0.5μg/ml, with only 24% of ac^+ DCs becoming $CD86^{hi}$ compared with 46% of ac^-DCs (Fig 4.2). Mature DCs were heterogeneous for CD54 with distinct populations of $CD54^{lo}$ and $CD54^{hi}$ cells, becoming most apparent at the highest dose of LPS (0.5μg/ml). Despite the general increase in fluorescent intensity of the ac^+ DCs after phagocytosis it is apparent that the percentage of $CD54^{hi}$ cells was lower in ac^+ than ac^- populations (Fig 4.2). CD40 expression was unaffected by apoptotic cells, with maturation in response to 0.5μg/ml generating a single $CD40^+$ population (Fig 4.2) and no statistically significant difference was seen in MHCII or CD80 expression. Hence the failure to upregulate CD86 in the ac^+ DCs was not due to

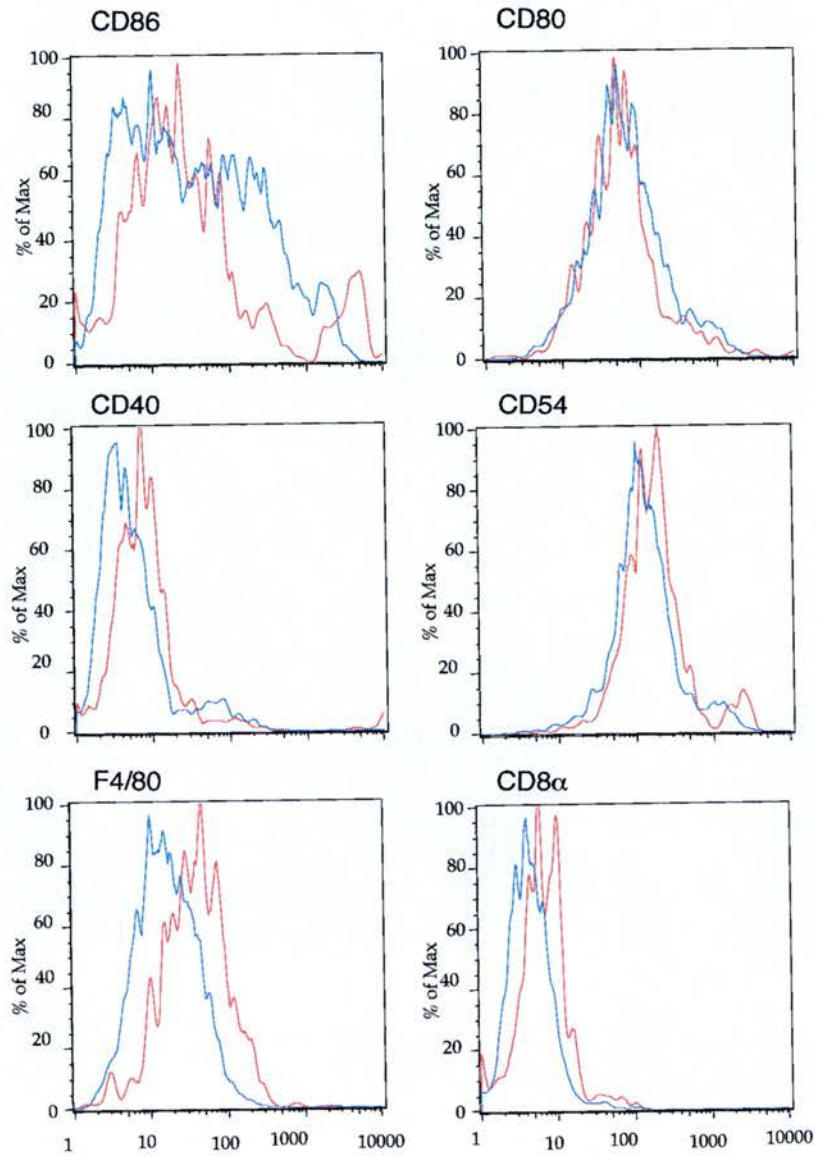


Figure 4.1:Cell surface phenotype of phagocytic vs nonphagocytic DCs. DCs were incubated with fluorescent apoptotic cells for 2 hours, and then stained for cell surface markers and analysed by FACS. Phagocytosing (red lines) and non-phagocytosing (blue lines) cells were identified by incorporation of green fluorescence from apoptotic cells. Both populations have similar levels of cell surface molecules, although phagocytosing DCs express slightly lower levels of CD86 and higher levels of F4/80 suggesting a slightly more immature phenotype

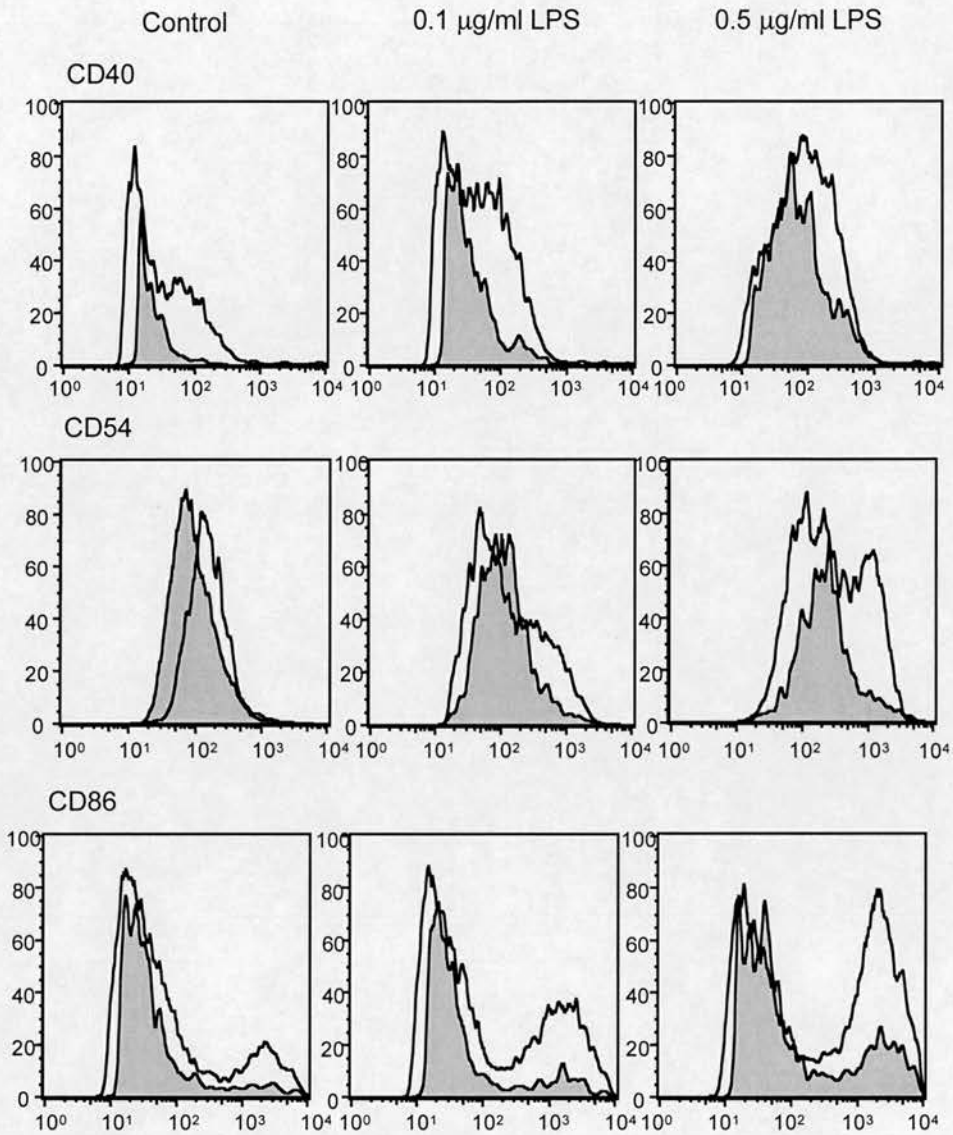


Figure 4.2:Cell surface phenotype of phagocytic vs nonphagocytic DCs after LPS maturation. DCs were incubated with fluorescent apoptotic cells for 2 hours, matured with LPS for 24 hours, stained for cell surface markers and analysed by FACS. Phagocytosing (filled histogram) and non-phagocytosing (open histogram) cells were identified by incorporation of green fluorescence from apoptotic cells. DCs containing apoptotic cells are less efficient at upregulating CD86 and to a lesser extent CD54 upon stimulation with LPS. In contrast no statistically significant difference was apparent in CD40 expression.

general unresponsiveness to LPS but appeared to affect a subset of costimulatory molecules.

Failure to upregulate CD86 expression in ac^+ DCs could reflect preferential ingestion of apoptotic cells by a subpopulation of DCs destined not to become $CD86^{hi}$ in response to maturation stimuli. However, when the mean CD86 fluorescence for the whole DC population was compared between DCs matured in the presence or absence of apoptotic cells (Fig 4.3 and 4.4), the presence of apoptotic cells resulted in a significantly lower CD86 fluorescence for the whole DC population compared to control. No such difference would have been detectable had the capacity to ingest apoptotic cells merely marked a subpopulation of DCs destined not to upregulate CD86 in response to LPS. This, and the observation that no significant difference in costimulatory molecule expression was detected between the ac^+DCs and ac^-DC population immediately after ingestion (Fig4.1) support the fact that phagocytosis had not preferentially occurred in a subpopulation destined to be $CD86^{lo}$. Also, this inhibitory effect was not merely a result of particle ingestion as immature DCs, when cocultured with latex beads and oxidised lipid, showed remarkably high levels (>85%) of phagocytosis and endocytosis respectively and exhibited no defect in LPS-driven upregulation of CD86 expression, exhibiting instead apparently enhanced expression (Fig 4.4.c). Taken together these data support the hypothesis that apoptotic cell ingestion alters DCs subsequent response to maturation stimuli.

4.2.2 Ingestion of apoptotic cells modulates proinflammatory cytokine secretion by DCs.

Cytokines produced by DCs are especially important in determining subsequent T cell responses. We therefore examined the effect of ingestion of apoptotic cells on cytokine production by DCs by combining the fluorescent phagocytosis assay and intracellular cytokine staining of cells, allowing us to study

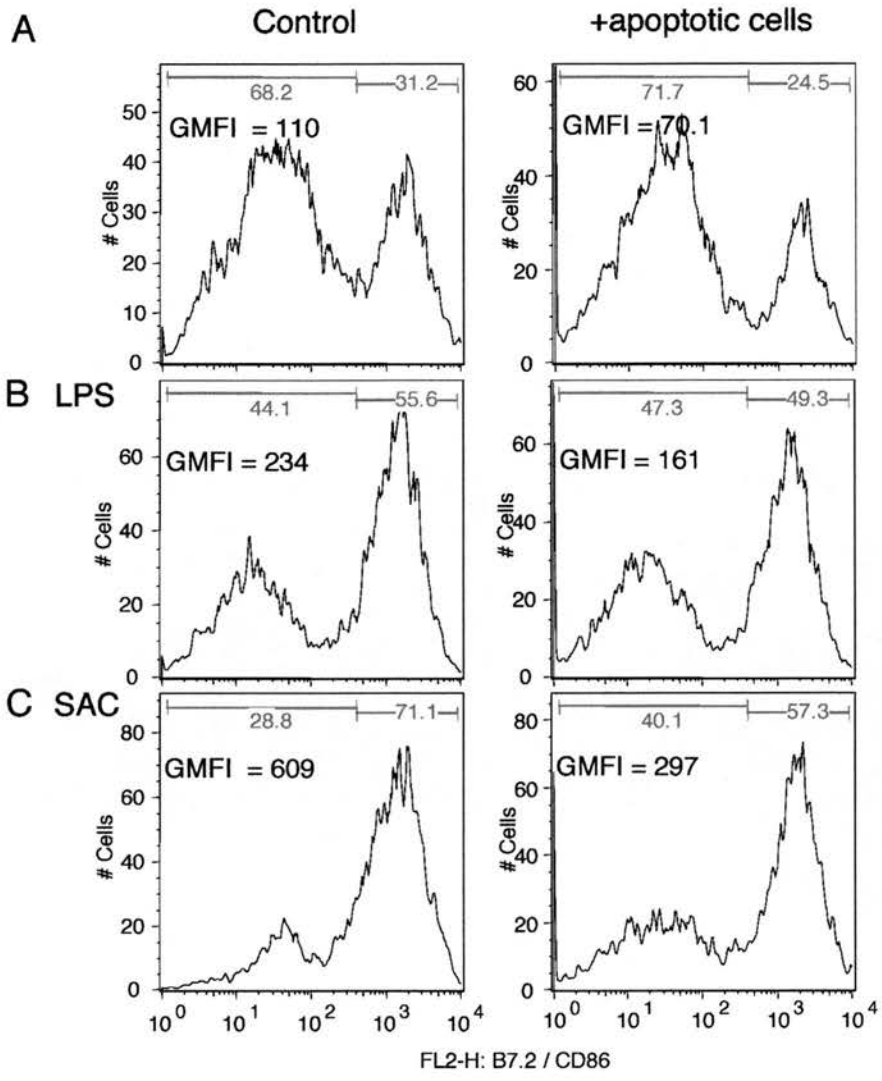


Figure 4.3: Effect of phagocytosis on DC upregulation of CD86 (1). DCs were incubated with apoptotic cells, and subsequently not stimulated (A) or matured with LPS (B) or SAC (C). Surface CD86 expression on DCs was determined by FACs. Apoptotic cells were excluded from analysis by size and bright green fluorescence. CD86 GMFI and percentage CD86 low and CD86 high are shown.

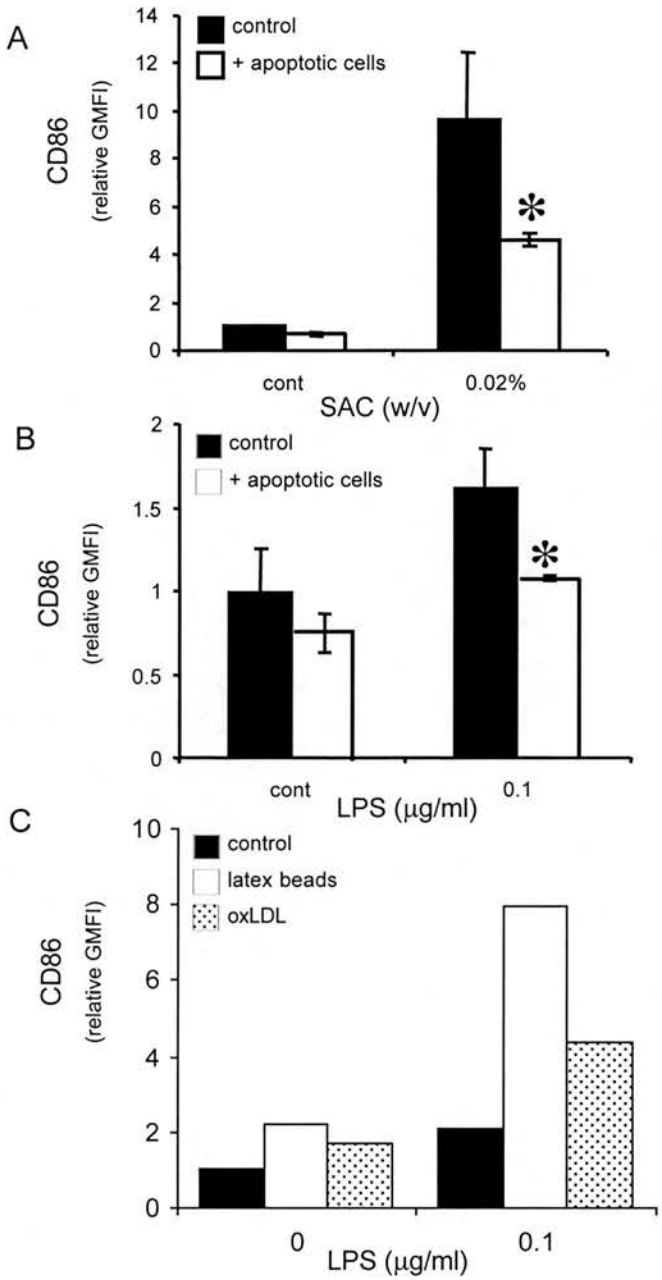


Figure 4.4: Effect of phagocytosis on DC upregulation of CD86 (2). A,B: DCs were incubated with apoptotic cells, and subsequently matured with SAC (A) or LPS (B). Surface CD86 expression on DCs was determined by FACs and is plotted as mean GMFI (relative to control unstimulated DCs) +/- sd. for 3 measurements from one culture. Similar results were seen in 3 (SAC) or 5 (LPS) experiments. *, $p< 0.05$ (by ANOVA).. Apoptotic cells were excluded from analysis by size and bright green fluorescence. **C:** DCs were also incubated with latex beads or oxLDL, and CD86 expression measured. The mean fluorescence of duplicate cultures is shown, with similar results seen in 2 independent experiments.

production of cytokines by individual DCs. Autocrine response to TNFα produced after LPS stimulation is an important factor in terminal maturation and activation of DCs as well as recruitment and activation of neighbouring effector cells. Interestingly, virtually all of the DCs containing apoptotic cells expressed TNFα after stimulation with LPS for 5 hours, demonstrating their functional viability and continuing responsiveness to LPS stimulation (Fig 4.5). A small population of the ac^- DCs failed to produce TNFα and probably represented a population of fully matured or 'exhausted' DCs (Fig 4.5). IL-12 is produced predominantly by DCs and orchestrates both the innate and adaptive immune response. DCs express a functional IL-12 receptor, ligation of which by bioactive IL-12p70 augments LPS maturation. In contrast to TNFα, ac^+ DCs failed to express IL12, even when stimulated with 0.5μg/ml of LPS (Fig 4.6). The inhibition of IL12 was further confirmed by ELISA of the supernatants and demonstrated a significant decrease in IL12 production by DCs co-cultured with apoptotic cells prior to LPS stimulation (Fig4.7). Interestingly, when DCs were stimulated by whole *Staph. aureus* apoptotic cells only appeared to inhibit IL12 produced at early time points (Fig4.8). This observation may reflect the ability of *Staph. aureus* to ligate a different TLR (TLR2) or the use of whole bacteria which might provide a more complex maturation stimulus.

4.2.3 Ingestion of apoptotic cells generates DCs with diminished capacity to sustain antigen dependent naive T cell proliferation and primes T cells for deletion.

To determine whether these cytokine and surface CD86 differences reflected a distinct functional phenotype of DCs, we chose to examine the capacity of ac^- DCs and ac^+ DCs to sustain antigen dependent naïve T cell proliferation, a process critically dependent on IL12 production and expression of costimulatory molecules. The use of unprimed T cells from the DO11.10 TCR transgenic mice

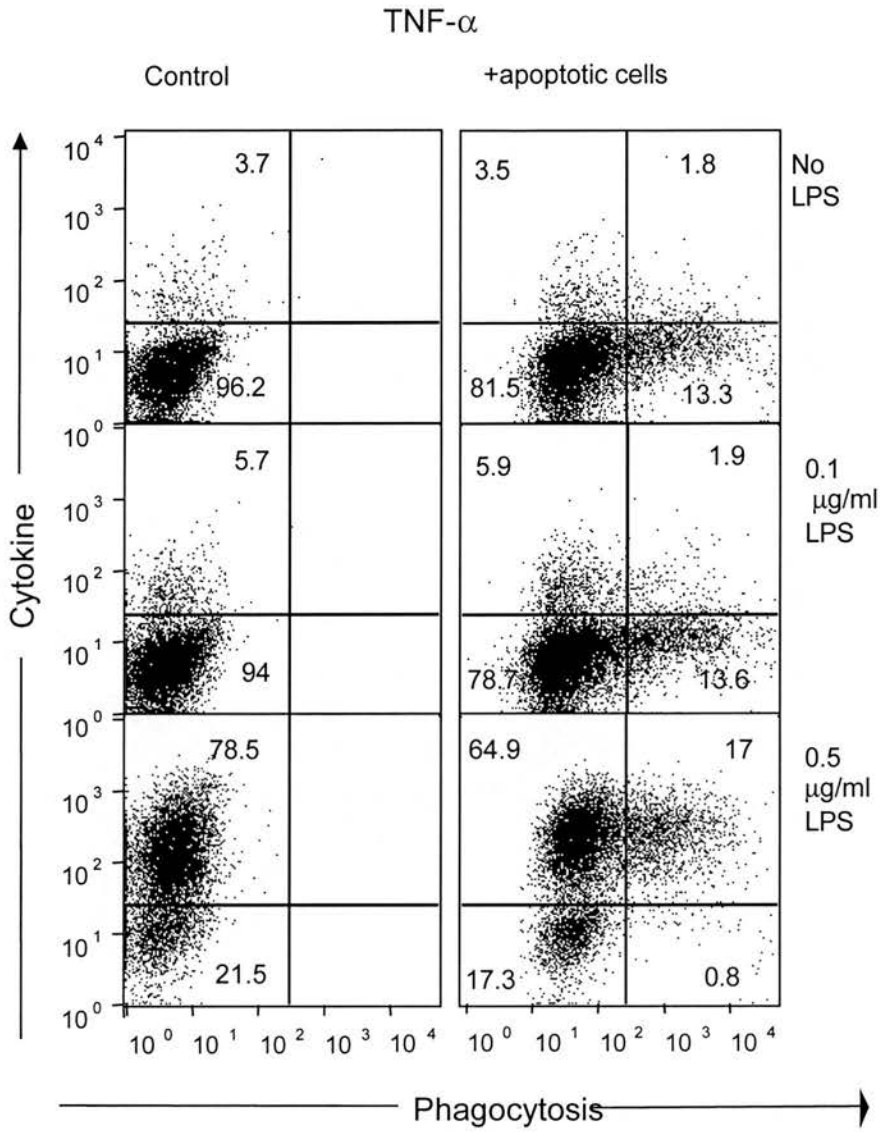


Figure 4.5: TNF-α expression by DCs following apoptotic cell phagocytosis. DCs were incubated with fluorescent apoptotic cells and stimulated with LPS. Intracellular TNF-α was measured after 4 hours by FACS analysis gated on CD11c+ cells. DCs that have ingested apoptotic cells can be distinguished on the basis of green fluorescence. Quadrant markers are set on DCs stained with isotype control antibodies (TNF-α) or DCs without apoptotic cells (phagocytosis). Figures give the percentage of cells in each quadrant. Data are representative of three independent experiments.

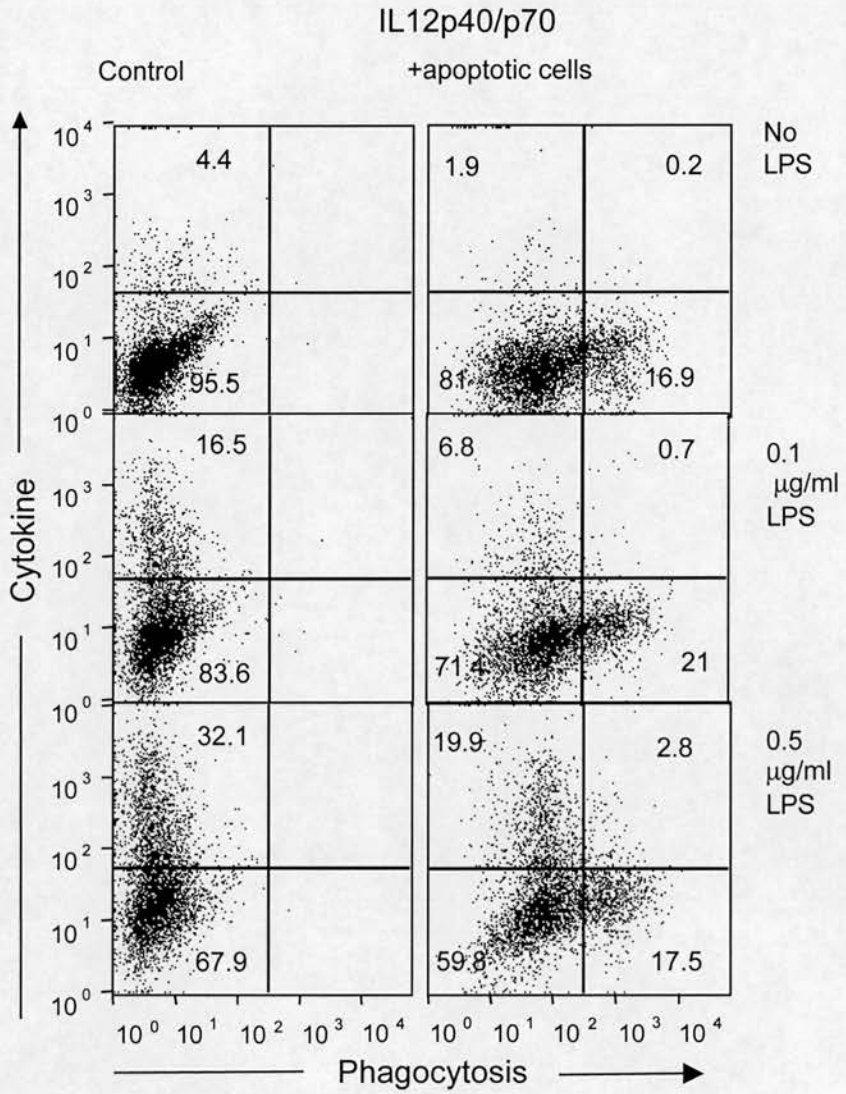


Figure 4.6: IL12 expression by DCs following apoptotic cell phagocytosis. DCs were incubated with fluorescent apoptotic cells and stimulated with LPS. Intracellular IL12p40/p70 was measured after 4 hours by FACS analysis gated on CD11c+ cells. DCs that have ingested apoptotic cells can be distinguished on the basis of green fluorescence. Quadrant markers are set on DCs stained with isotype control antibodies (IL12) or DCs without apoptotic cells (phagocytosis). Figures give the percentage of cells in each quadrant. Data are representative of three independent experiments.

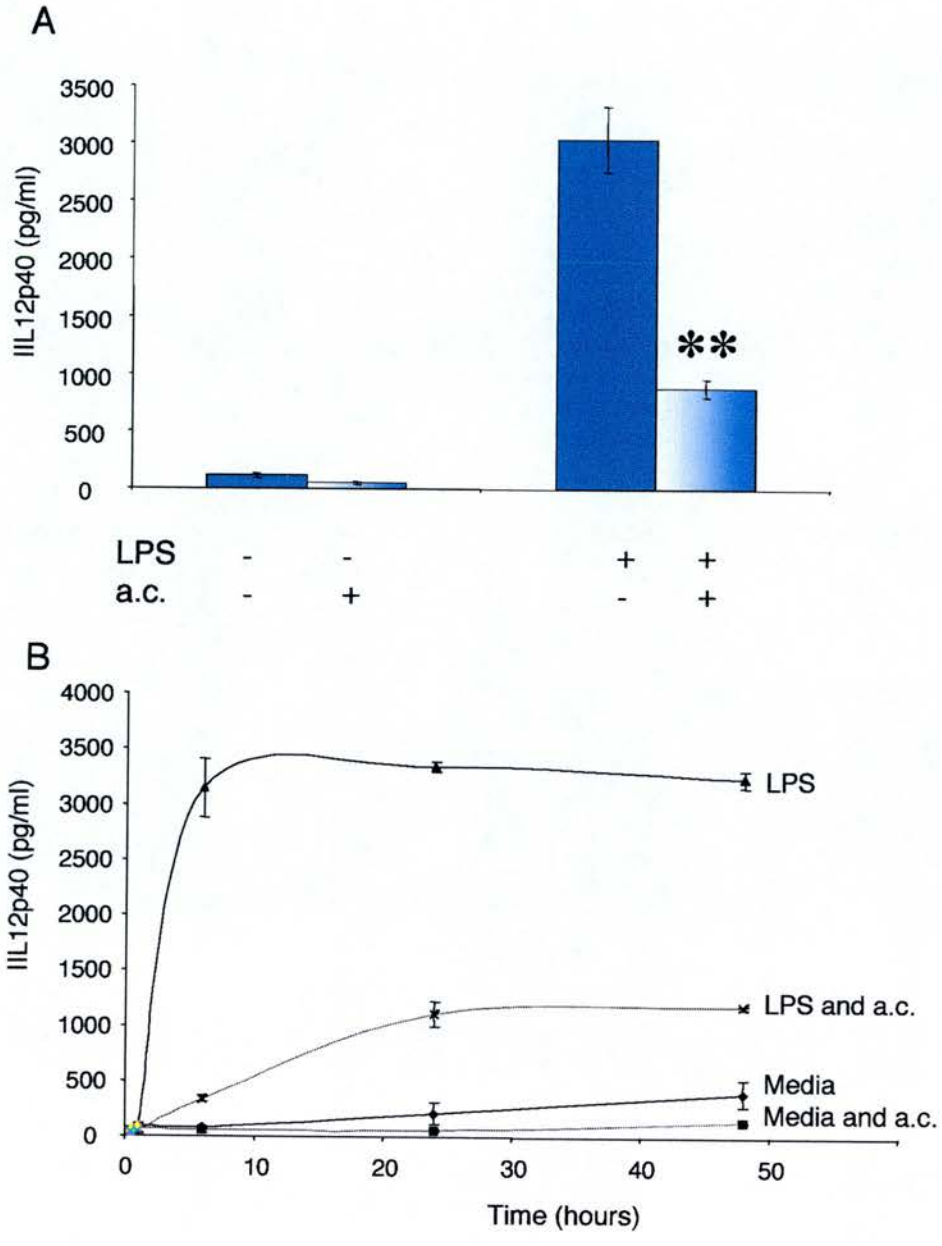


Figure 4.7: IL12 production by DCs following apoptotic cell phagocytosis and LPS stimulation. DCs were incubated with apoptotic cells and stimulated with LPS. Culture supernatants were harvested at 1 to 48 hours and levels of IL12 measured by ELISA. Data are presented as mean +/- s.d. of triplicate measurements for 6 hour supernatants (**A**) or the full time course (**B**). LPS stimulated IL12 production was inhibited at all time. Similar results were seen in 2 experiments.

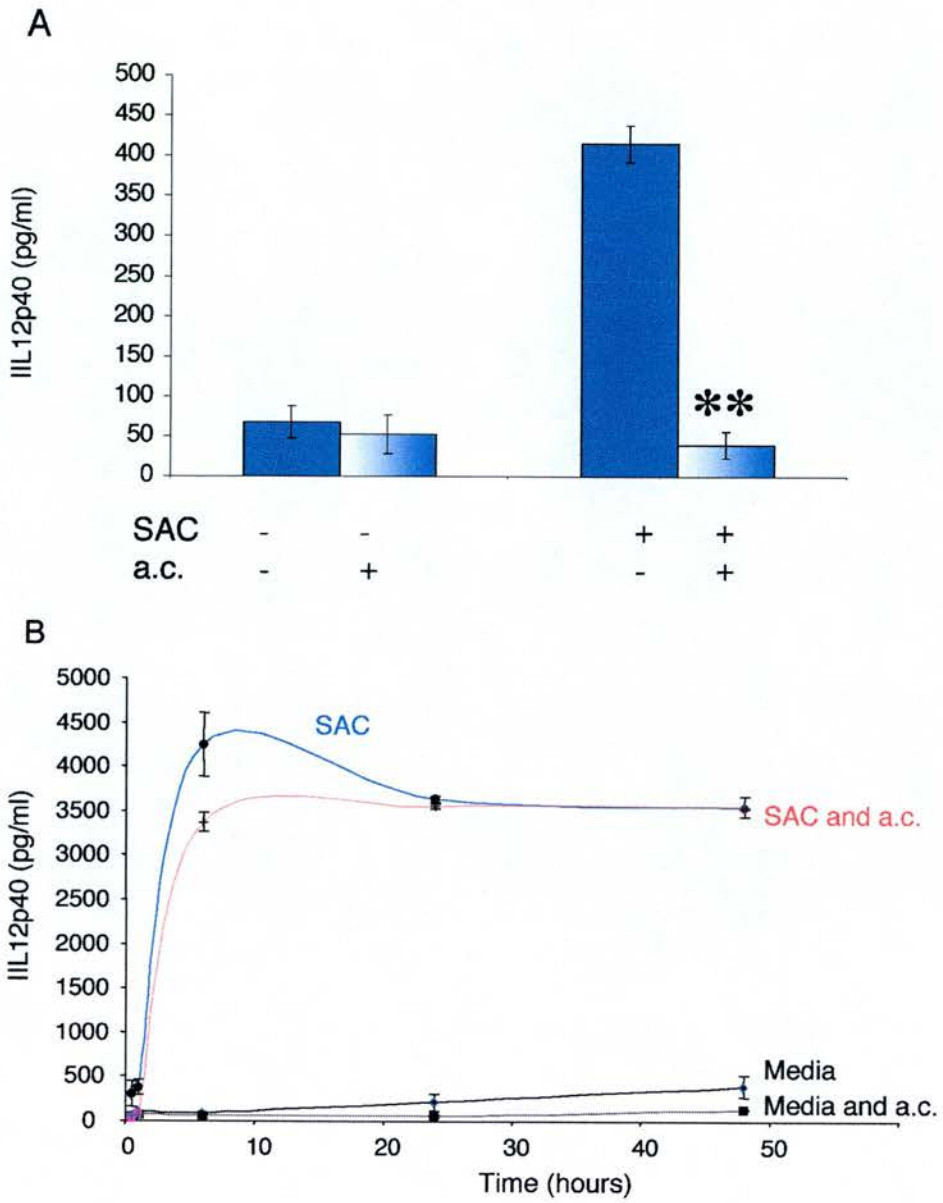


Figure 4.8: IL12 production by DCs following apoptotic cell phagocytosis and SAC stimulation. DCs were incubated with apoptotic cells and stimulated with SAC. Culture supernatants were harvested at 1 to 48 hours and levels of IL12 measured by ELISA. Data are presented as mean +/- s.d. of triplicate measurements for 1 hour supernatants (**A**) or the full time course (**B**). SAC stimulated IL12 production was only inhibited at 1-6 hours. Similar results were seen in 2 experiments.

allowed us directly to compare T cell proliferation in response to mature ac$^+$ DCs vs ac$^-$ DCs, pulsed in both cases with $OVA_{323-339}$ peptide after LPS maturation. Interestingly ac$^+$ DCs retained the ability to sustain naïve T cell proliferation but were only approximately 30% as effective as stimulators when compared to ac$^-$ DCs or DCs matured without apoptotic cells when cultured at a ratio of 10:1 T cells : DCs (Fig 4.9).

Both IL12 and costimulatory molecule expression are known to be important not only in the amount of T cell stimulation delivered but also in determining the outcome of the interaction. Thus the supernatants were examined to determine if either ac$^+$ or ac$^-$ DCs preferentially drove Th1 or Th2 cytokine production. Interestingly, both ac$^+$and ac$^-$ DCs stimulated IFNγ production by T cells (Fig4.10) and only negligible amounts of IL4. Furthermore, PMA/ionomycin restimulation of T cells after a week in culture showed no increase in Th2 cytokine production when examined by intracellular cytokine staining with both ac$^+$and ac$^-$DCs producing T cells that secrete IFNγ. However, upon PMA/ionomycin restimulation, ac$^+$DC primed T cells initially proliferated and then underwent accelerated activation induced cell death such that, after 24 hours of stimulation, more than 75% of the T cells were apoptotic compared to only 25% of ac$^-$ or control DC primed T cells (Fig4.11).

4.2.4 'Necrotic' cells

Cells dying by apoptosis are rapidly cleared and hence secondarily necrotic cells, which have lost membrane integrity after undergoing apoptosis and begun to 'leak' intracellular contents, are uncommon *in vivo* unless apoptotic cell clearance is impaired. It is postulated that clearance of such secondary necrotic cells may be associated with autoimmunity. This process of secondary necrosis can be recapitulated *in vitro* by ageing neutrophils for longer periods, a process associated with increased numbers of anucleated 'late-apoptotic cells'

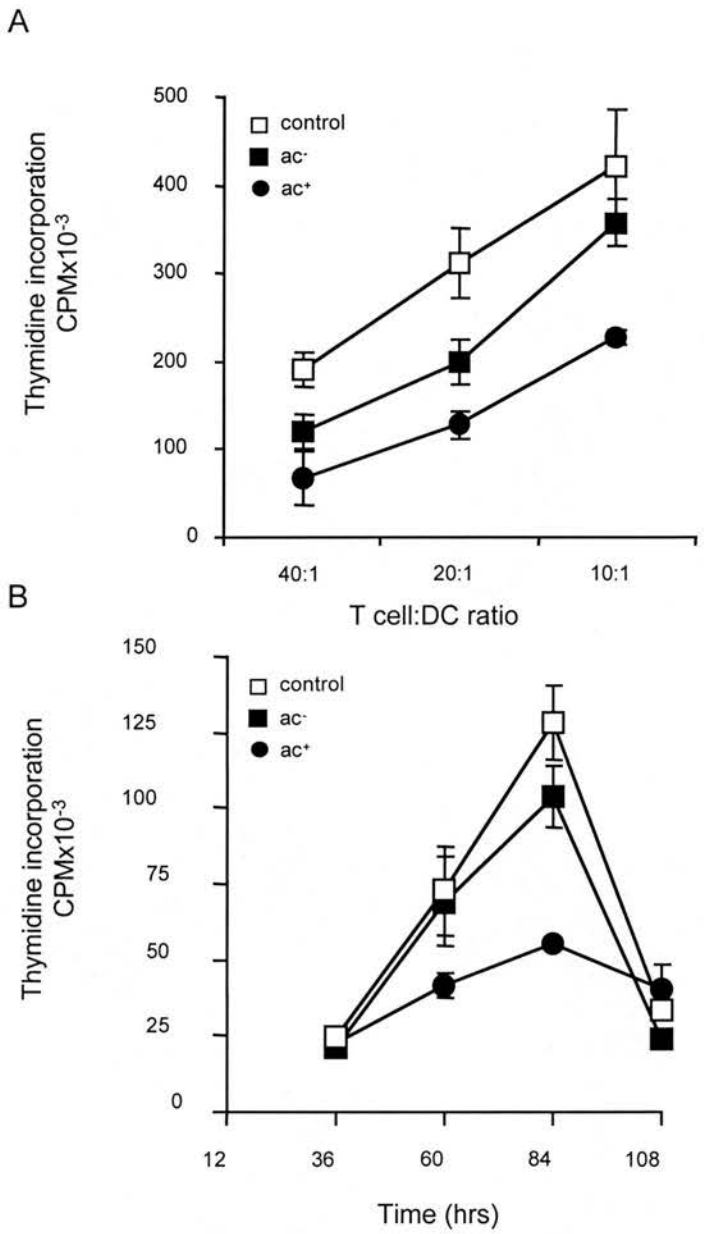


Figure 4.9: Antigen-driven T cell stimulation by DCs following apoptotic cell phagocytosis. Mature DCs were pulsed with $OVA_{232-339}$ peptide, sorted into ac- and ac+ cells and incubated with T cells from DO11.10 mice. DCs that had not been incubated with apoptotic cells were included as a control. T cell proliferation was measured by [^{3}H]-thymidine incorporation. **A**: Proliferation in response to different T cell: DC ratios, measured after 3 days. **B:** Time course of T cell/ DC interaction demonstrates inhibition at all time points. Background proliferation in the absence of OVA peptide was < 2000 cpm in all experiments. The mean +/- s.d. from triplicate measurements in one experiment representative of three independent experiments are shown.

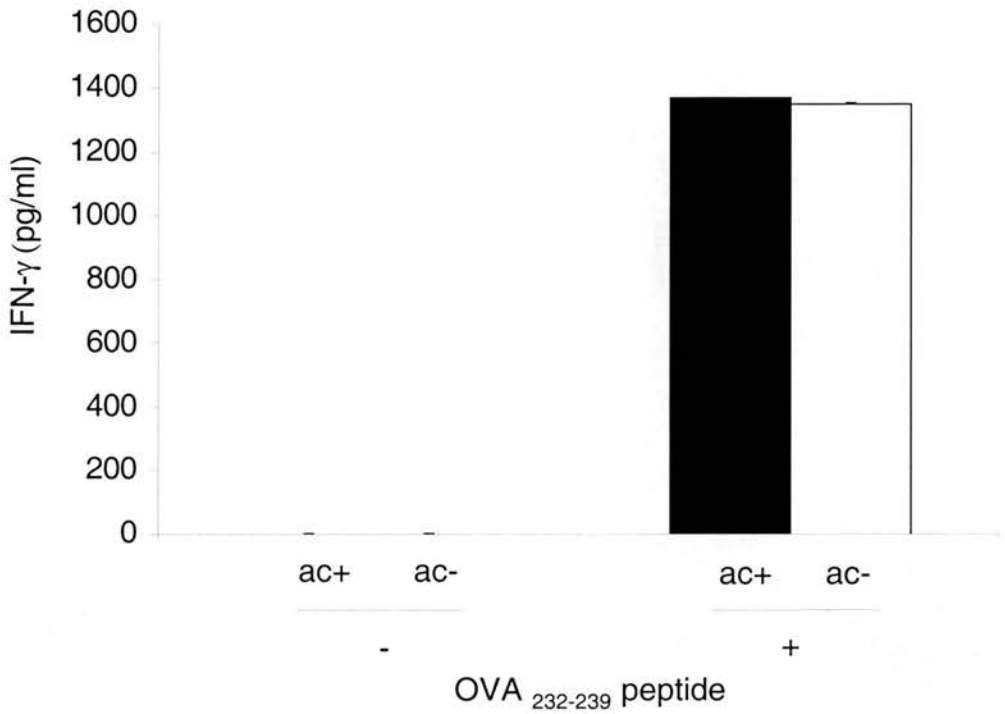


Figure 4.10: IFN-γ production by ac+ and ac- DC stimulated T cells. Mature DCs were pulsed with $OVA_{232-339}$ peptide, sorted into ac- and ac+ cells and incubated with T cells from DO11.10 mice. Supernatants from T cell cultures were harvested and levels of IFN-γ measured by ELISA at 5 days.

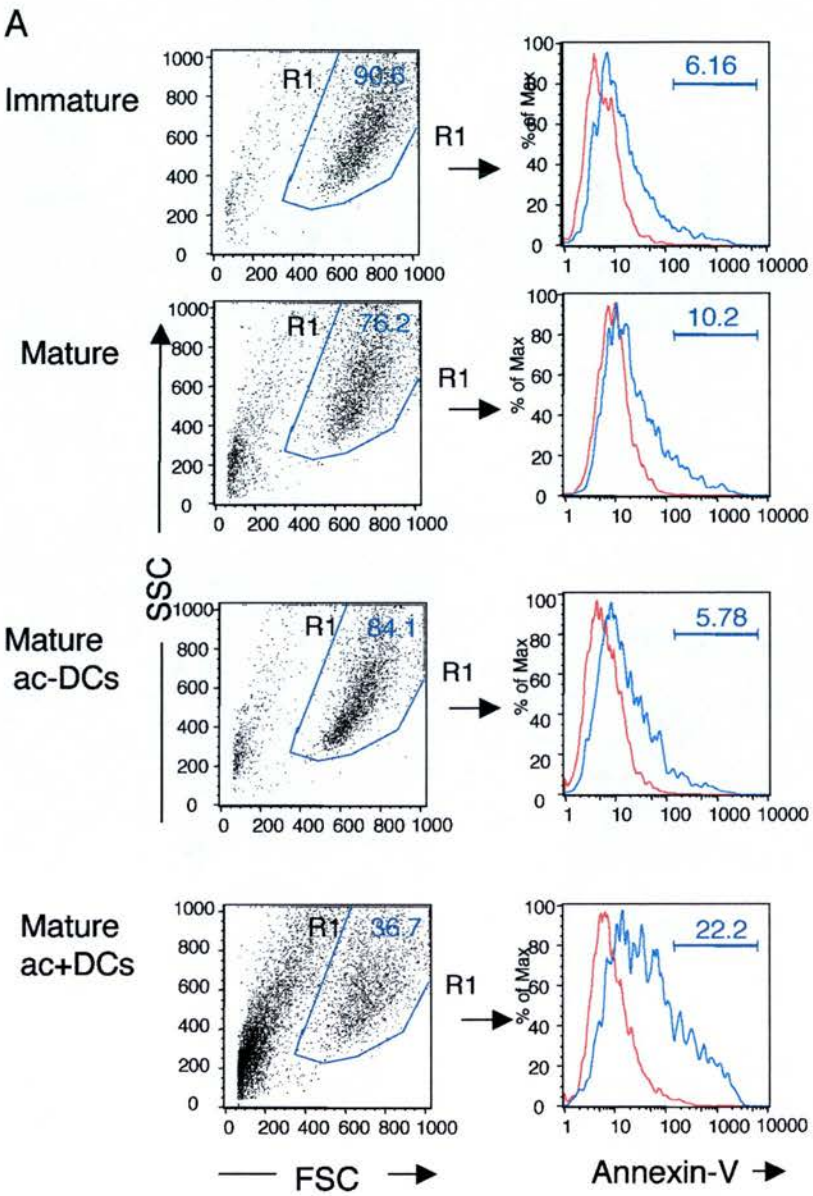


Figure 4.11: ac+ DCs prime T cells for death upon restimulation (A). T cells from DO11.10 mice were incubated with immature and mature DCs that were sorted into ac- and ac+ cells and pulsed with $OVA_{232-339}$ peptide. T cells were then restimulated with PMA and ionomycin, and analysed for apoptotic cells by size and Annexin V staining.

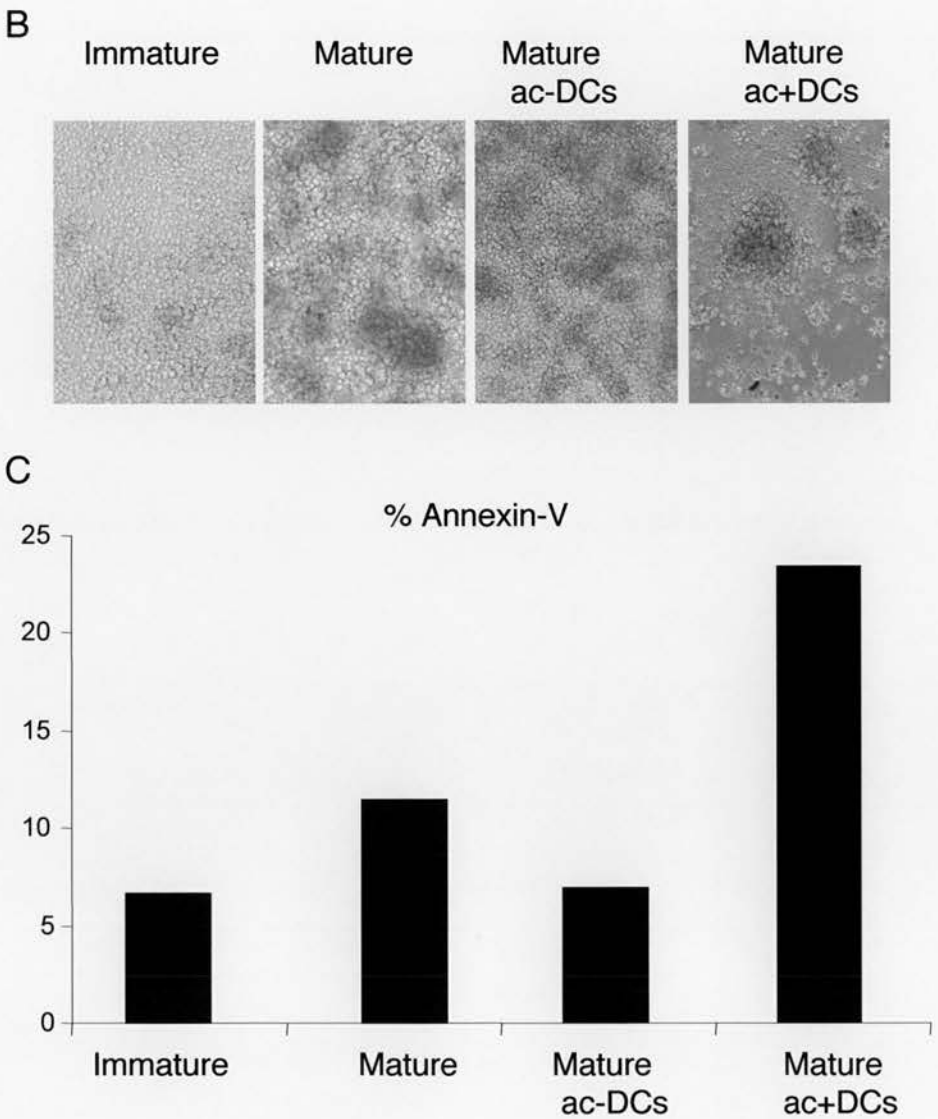


Figure 4.11: ac+ DCs prime T cells for death upon restimulation (B, C). T cells from DO11.10 mice were incubated with immature and mature DCs that were sorted into ac- and ac+ cells and pulsed with $OVA_{232-339}$ peptide. T cells were then restimulated with PMA and ionomycin. **B**: Photographs of restimulated cells. **C:** Percentage of cells gated as 'live' by forward/ side scatter that are Annexin V positive, hence apoptotic.

(Ren et al. 2001). These cells demonstrate an intermediate level of permeability to PI and are annexin positive when compared to cells rendered necrotic by heat treatment. Previous experiments in our lab have demonstrated that macrophage clearance of such late-apoptotic cells is in fact non-phlogistic (Ren et al. 2001). To determine whether inhibition of DC activation was only a feature of early apoptosis we cultured DCs with heat-treated 'necrotic' cells (representative of the terminal stage of cell death). DCs efficiently internalized these 'necrotic' cells a process surprisingly associated with inhibition of IL12 (Fig 4.12) as seen with apoptotic cells (Fig 4.13). Furthermore, by intracellular staining, these 'necrotic cells' also appeared to inhibit TNFα production to some degree (Fig 4.14).

4.3 Discussion

The data presented demonstrate that DC ingestion of apoptotic cells, but not control particles, results in down regulation of LPS driven IL12 production and CD86 expression. Furthermore this correlated with impaired antigen dependent T cell activation *in vitro* and also appeared to prime T cells for deletion on subsequent stimulation. Interestingly, these effects were restricted to those DCs that had ingested apoptotic cells and were also seen after internalization of our *in vitro* generated 'necrotic' cells implicating ligation of specific phagocytic receptors, recognising 'altered self' motifs present on the cell surface of dead cells, in this process.

Apoptotic cells are poorly immunogenic and, unless they overload normal clearance mechanism or are associated with danger signals, rarely incite an immune response. Furthermore, UV irradiation, characterized by widespread apoptosis, is associated with generalised immunological hyporesponsiveness (Yoshida et al. 1998), suggesting a potential immunosupressive effect of

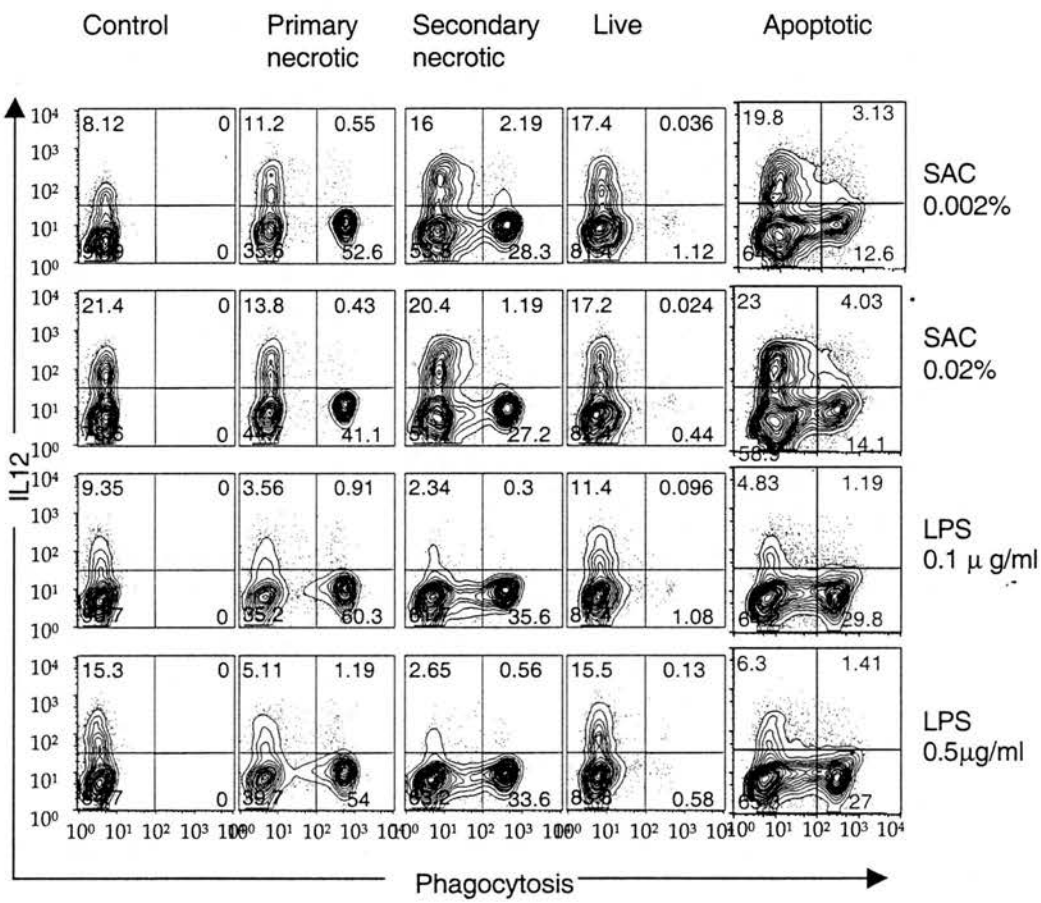


Figure 4.12: Effects of different 'dying' cells on IL12 expression by DCs. DCs were incubated with fluoresecntly labelled untreated (live), apoptotic and heat treated cells and stimulated with LPS. IL12 expression was measured by intracellular cytokine staining, analysed by FACS and is plotted against phagocytosis. Both primary heat treated live cells and secondarily necrotic (heat treated apoptotic cells) are efficiently internalised by DCs and inhibit the production of IL12 in response to LPS and SAC. Live cells are not internalised by DCs.

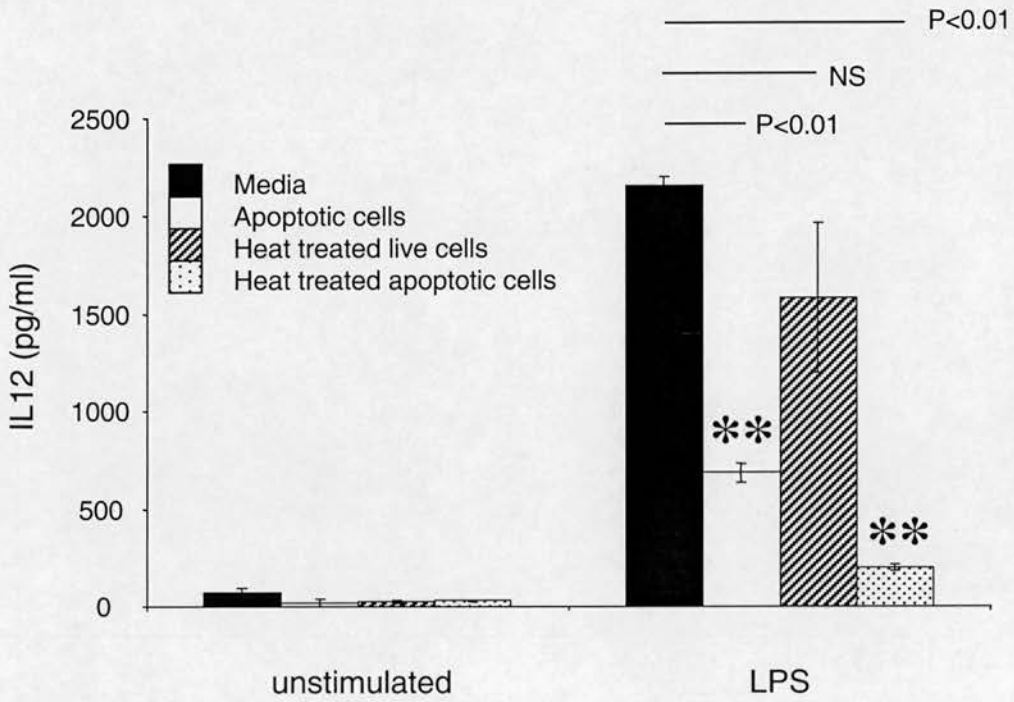


Figure 4.13: Effects of different 'dying' cells on IL12 production by DCs. DCs were incubated with untreated (live), apoptotic and heat treated cells and stimulated with LPS. Cell culture supernatants were harvested and levels of IL12 measured by ELISA. Apoptotic and heat treated apoptotic cells (secondarily necrotic) primary cells inhibit LPS driven IL12 production to a greater extent than heat treated live (primarily necrotic) cells.

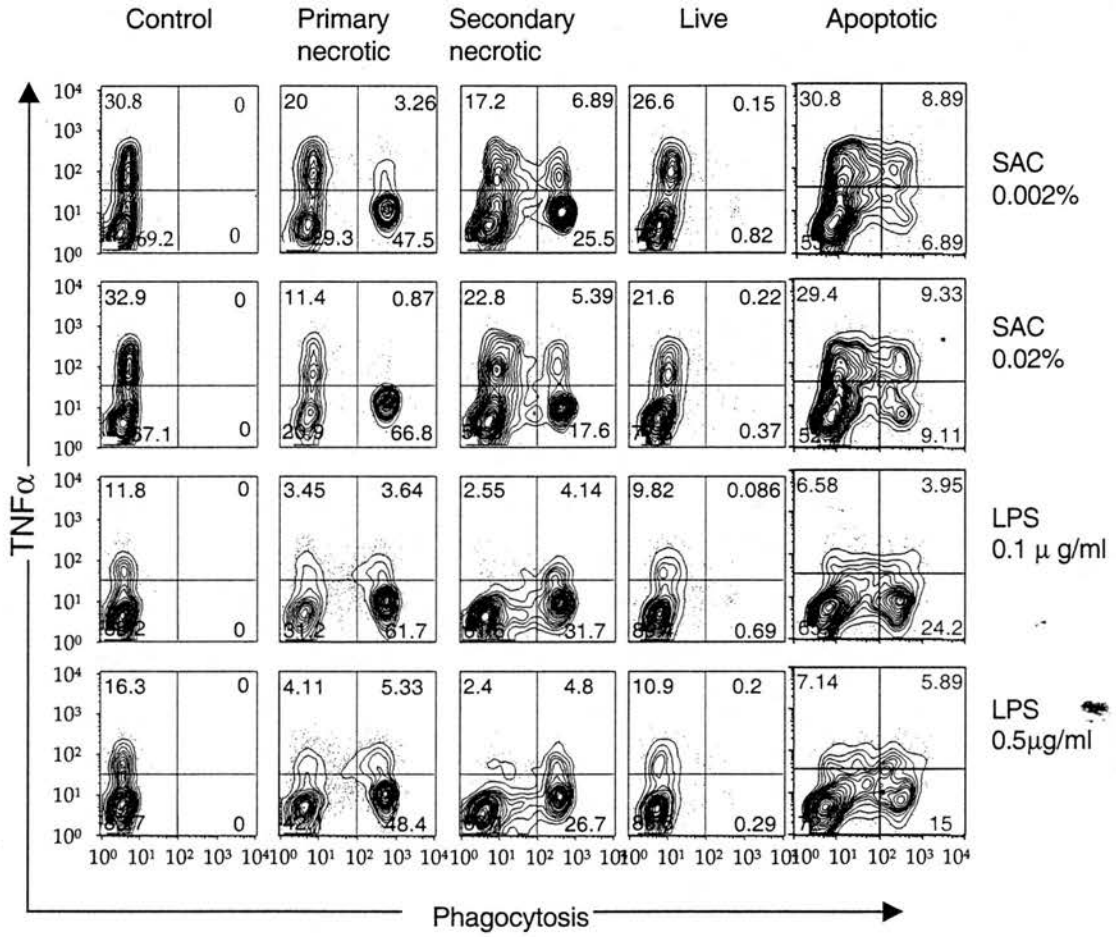


Figure 4.14: Effects of different 'dying' cells on TNF-α expression by DCs. DCs were incubated with fluoresecntly labelled untreated (live), apoptotic and heat treated cells and stimulated with LPS. TNF-α expression was measured by intracellular cytokine staining, analysed by FACS and is plotted against phagocytosis.

apoptotic cells on the adaptive immune system. In two interesting recent reports, apoptotic cells have been shown to be capable of promoting transplantation tolerance (Bittencourt et al. 2001) and protecting from autoimmunity (Hugues et al. 2002). Although the exact mechanisms of immunosuppression in these systems were not fully understood, a bone marrow derived cell, likely to be the DC, has been implicated. Exactly how this occurs is controversial but an increasingly accepted view is that the immature DC, with low levels of costimulatory molecule expression, would fail to deliver 'signal two' and induce anergy or deletion of an interacting T cell. In support of this, repeated immunisation with immature DCs does appear to induce Treg/Tr1 cells (Dhodapkar, M. V. et al. 2001). In contrast, mature DCs, which are able to secrete a potent stimulatory cytokine, IL12, and express high levels of costimulatory molecule expression, induce strong adaptive immunity. Interestingly, the observation that ac^+ DCs appear to prime T cells for deletion upon restimulation is supported by other studies; for instance, *in vivo* cross-priming to cellular antigen has been shown to result in deletion of antigen specific CD8 T cells (Kurts et al. 1997a; Kurts et al. 1997b) and, in a similar human *in vitro* model, deletion of influenza specific $CD8^+$ T cells also occurred unless CD40 ligation was provided to the DC (Albert et al. 2001). Thus, although the mechanism of deletion in these class I restricted systems is not clear, it is possible that such ac^+DCs prime not only CD8 but also CD4 T cells for deletion. One possible contributing factor might be the failure of ac^+ DCs to produce IL12 whilst continuing to produce TNFα. IL12 has been shown to have a myriad of functions including modulating Th1 vs Th2 switching, activation of NK cells and production of IFN-γ. Furthermore, autocrine effects of IL12 have been shown to augment DC responses to exogenous stimuli, underscoring the importance of this cytokine in DC effector functions and the subsequent adaptive immune response (Adorini et al. 1996; Lamont et al. 1996). In addition, cytotoxic activity of DCs has been attributed to various members of the TNF superfamily (Liu, S. et al. 2001a; Vidalain et al. 2001; Yu et al. 2002), including TNFα and TRAIL, the former being secreted by ac^+DCs.

Of note, my experiments with necrotic cells yield data that contrast with some reported studies. Necrotic cells have been shown to have some proinflammatroy effects, promoting maturation of DCs perhaps through associated danger signals such as heat shock proteins or proteases (Basu et al. 2000). The process by which I rendered cells 'necrotic' may not represent necrosis found *in vivo* well and the associated 'danger signals' released from intracellular contents may have been destroyed during heat treatment. Furthermore, Sauter et al's rigorous examination of the potential of a variety of necrotic cells to promote maturation of DCs demonstrated that it was a feature only of certain immortalized or malignant cells and was not demonstrated by primary cells such as those used in these studies (Sauter et al. 2000). In addition, Fadok et al have demonstrated that the presence of PS bearing/apoptotic membrane fragments, as found in our 'necrotic' cells (Fig 2.1) was able to provide an inhibitory signal which, in their macrophage system, was dominant over any danger signals from necrotic cells (Fadok et al. 2001). Hence, the anti-inflammatory signal from the dying cell membrane may be overriding proinflammatory mediators in our system. Finally, in *C. elegans*, clearance of necrotic and apoptotic cells occurs by a shared phagocytic pathway (Chung et al. 2000) and may therefore stimulate similar inhibitory responses. It is possible that a phlogistic response to necrotic cells requires additional cues from the micro-environment such as activation of proteolytic cascades or tissue derived 'danger signals' that would not be apparent *in vitro*.

In conclusion, it is essential for DCs to mature before they can activate naïve T cells and these data and two recent studies confirm that ingestion of apoptotic cells alone did not provide sufficient maturation stimulus. Therefore, DCs ingesting apoptotic cells must be exposed to additional stimuli such as heat shock proteins, monocyte-conditioned medium or viral products before they become capable of stimulating T cells. Many of these agents will be present in inflamed sites alongside apoptotic cells *in vivo*, and the potential for DCs both to acquire apoptotic cell derived self-antigens and receive maturation signals is high. However autoimmunity is uncommon and the response of the DC is

likely to be tightly regulated. This data would suggest that ingestion of apoptotic cells is not immunologically null but is capable of regulating DC maturation, providing a counterbalance for inflammatory stimuli. A failure to see these inhibitory effects of apoptotic cells in other studies may reflect the percentage of DCs ingesting apoptotic cells, the strength of the maturation stimulus used, the mechanism of cell death or an inherent difference in response of DCs to constituitive vs. induced apoptosis. In the next chapters, we will attempt to investigate possible mechanisms by which such inhibitory effects are exerted on DCs and defining whether apoptotic cells act directly to alter DCs effector functions *in vivo* or whether interactions with other cells of the monocyte lineage is required.

CHAPTER 5: INHIBITORY EFFECTS OF APOPTOTIC CELLS ON DCS BOTH EX VIVO AND IN VIVO

5.1 Introduction

I was interested in whether my observations of DC inhibition by apoptotic cells would be restricted to *in vitro* bone marrow derived DCs or whether similar inhibitory effects might be seen in *ex vivo* or *in vivo* DC. In this chapter we will demonstrate that the effects of apoptotic cells are able to modulate *ex vivo* isolated DCs and also *in vivo* IL12 production.

5.2 Results

5.2.1 Isolation of *ex vivo* DCs

Dendritic cells exist at low frequency in tissue but are widely distributed in secondary lymphoid tissue such as the spleen and lymph nodes. The spleen contains three of the five DC subsets recognised in the mouse (Liu, Y. J. 2001; Shortman et al. 2002). An important DC subset, the CD8α+CD11c+DC constitutes approximately 25% of DCs within the spleen and have been shown to be the principal source of IL12 production *in vivo* and are responsible for cross-presentation of cell derived antigens. Using a protocol modified from C Reis e Sousa (Schulz et al. 2002a) it was possible to obtain highly enriched DC preparations (Fig5.1) containing all of the CD11c+ subsets described in the spleen (i.e. CD4+/CD8α+, CD4+/CD8α- and CD4-/CD8α- DCs) (Shortman et al. 2002). These DCs were viable and relatively unactivated upon isolation, matured spontaneously in overnight culture (Fig5. 1) and retained the capacity to respond to LPS stimulation (Fig 5.1).

5.2.2 *Ex vivo* DCs contain apoptotic cells

To assess the morphology of our *ex vivo* DCs we performed cytospins which demonstrate the characteristics of DCs (Fig5.2). Interestingly, some of the isolated DCs contained cytoplasmic inclusions with tight condensed nuclei

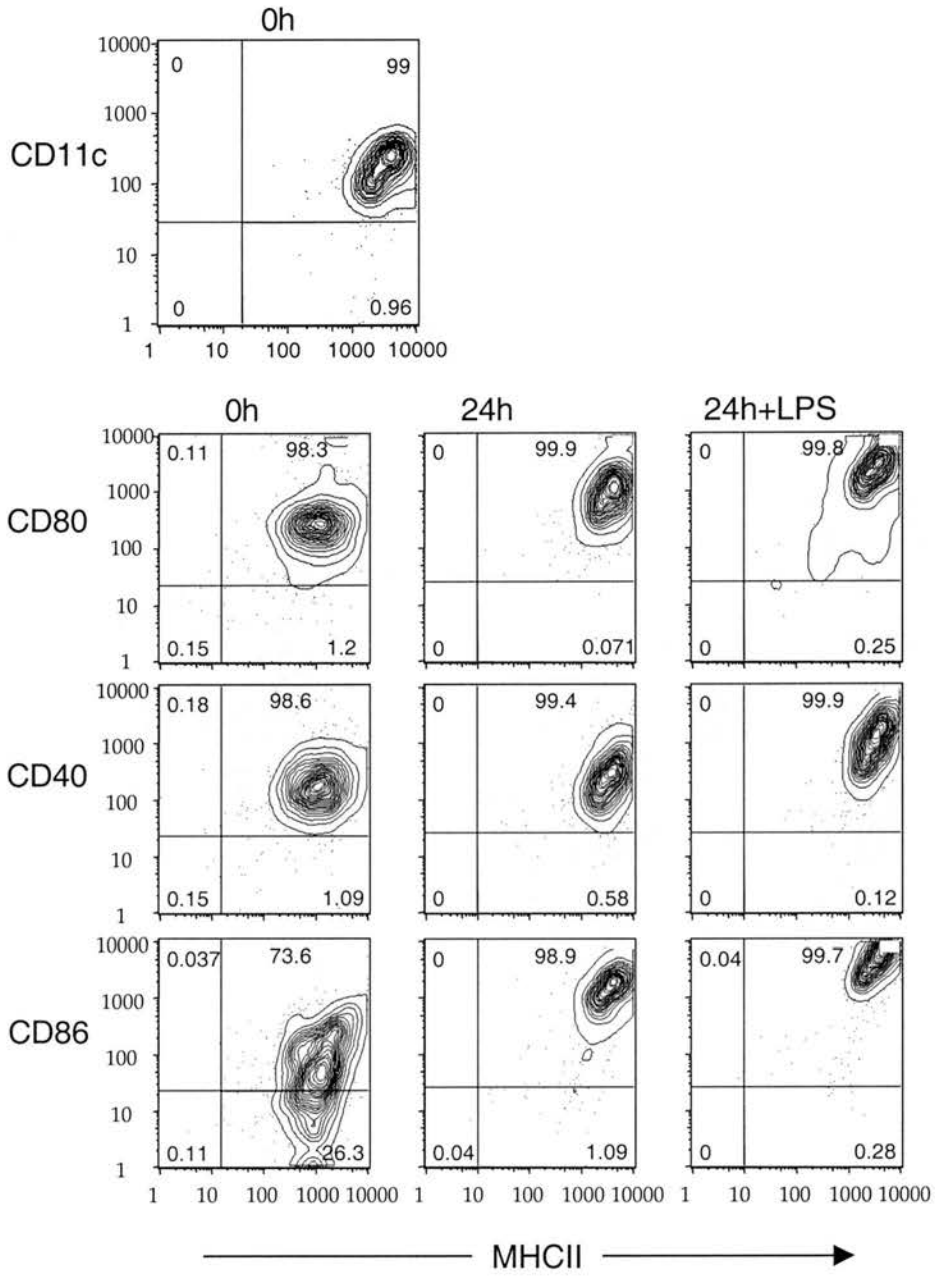


Figure 5.1: Cell surface phenotype of *ex vivo* isolated DCs. DCs were harvested from mouse spleen and purified by positive sorting for CD11c+ cells. DCs were stained for surface molecule expression after purification or cultured for a further 24 hours with or without LPS (1 μg/ml), and stained. DCs show some spontaneous maturation in culture (demonstrated by increases in CD80, CD40 and CD86 expression) which is augmented further by LPS.

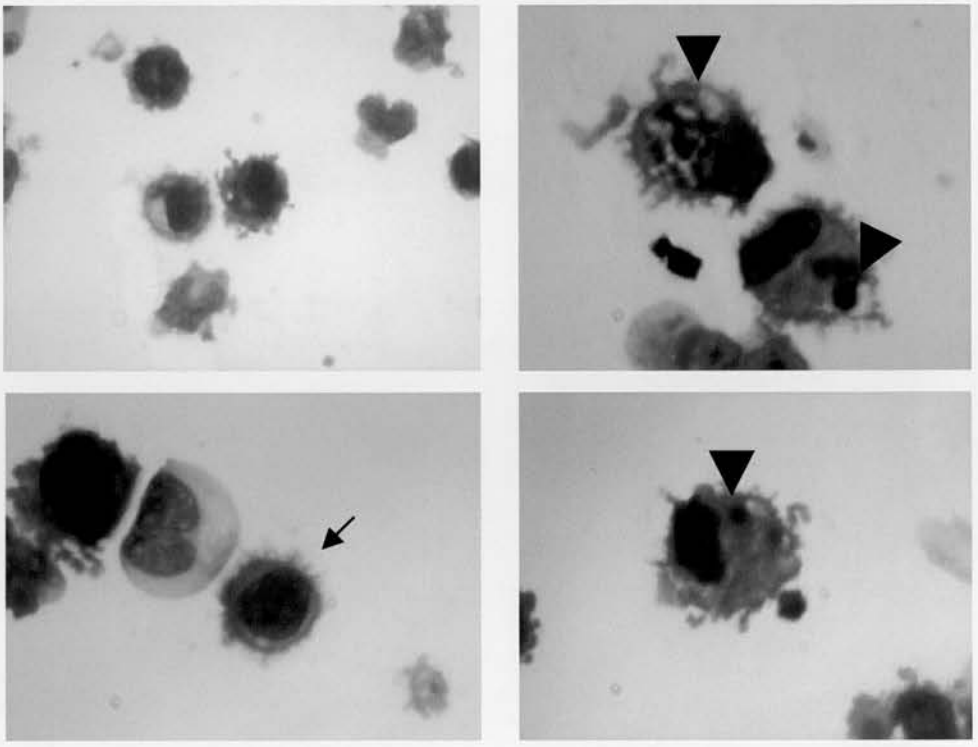


Figure 5.2: Cytospins of *ex vivo* isolated DCs. CD11c+ spleen DCs were cytospun, stained (Dif-Quik) and photographed under x 100 (oil) objective. DCs demonstrate fine cytoplasmic protrusions (arrow). Some DCs had blunt pseudopodia and contained prominent granular inclusions reminiscent of apoptotic bodies (arrowhead).

characteristic of apoptotic cells and apoptotic cells could also be seen contained in the phagolysosome of the DCs (Fig5.2).

5.2.3 *Ex vivo* DCs express scavenger receptor and phagocytose

The immediate *ex vivo* identification of DCs that appeared to contain newly phagocytosed apoptotic cells implied that these splenic DCs may retained their phagocytic capacity. Furthermore, I identified that these DCs expressed CD36, (Fig5. 3) which was found preferentially on CD8α+DCs, further suggesting that they would internalise apoptotic cells. Thus, to test the ability of these DCs to phagocytose apoptotic cells DCs were challenged with fluorescent apoptotic murine thymocytes or neutrophils, which were efficiently internalised (Fig5. 4). Interestingly at high apoptotic cell: DC ratios (40:1) CD8α-DCs also internalised apoptotic cells.

5.2.4 *Ex vivo* DCs are inhibited by apoptotic cells.

Bone marrow derived DCs share many characteristic with macrophages and the ability of apoptotic cells to inhibit cultured DCs in previous experiments might simply represent an *in vitro* artefact. Thus I chose to test the ability of freshly isolated splenic DCs to be inhibited by apoptotic cells. Culturing these DCs with apoptotic cells also inhibited LPS driven upregulation of CD86 (Fig5.5) in a similar manner to the murine bone marrow cultures, confirming that this characteristic was probably conserved between many DC types. Furthermore, IL12 production from whole splenocytes in response to LPS was also inhibited in a manner very similar to the bone marrow derived cells (Fig 5.6). Cell sorting of splenocytes into CD11c+ and CD11c- fractions confirmed previous reports that DCs are responsible for all IL12 produced and demonstrated that they were inhibited by apoptotic cells independent of other cell populations found in the spleen (Fig 5.7).

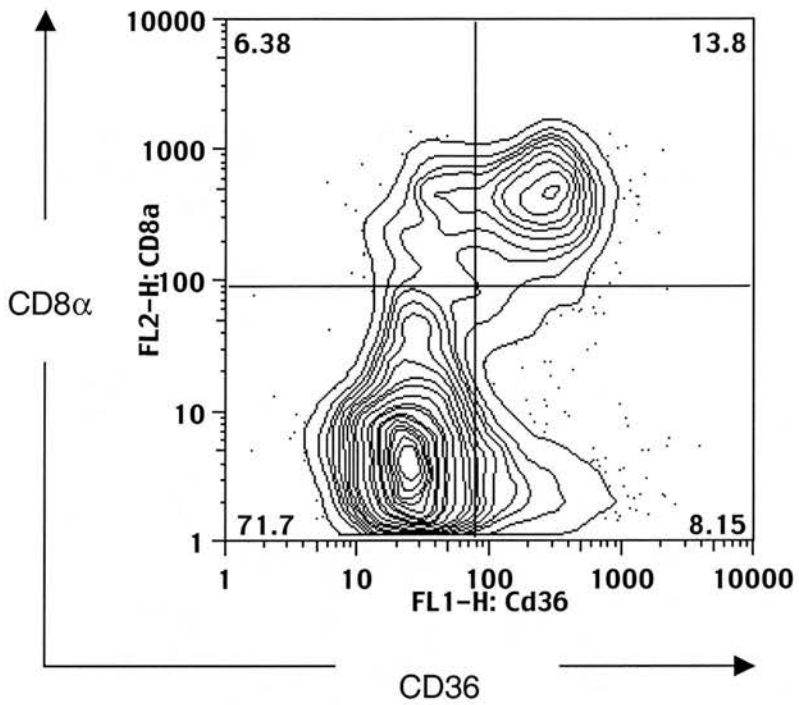


Figure 5.3: CD36 expression on *ex vivo* DCs. Mouse spleen cells were stained for CD11c, CD36 and CD8α. Figure shows that CD8α + cells express high levels of CD36. FACS analysis shown is gated on CD11c+ cells.

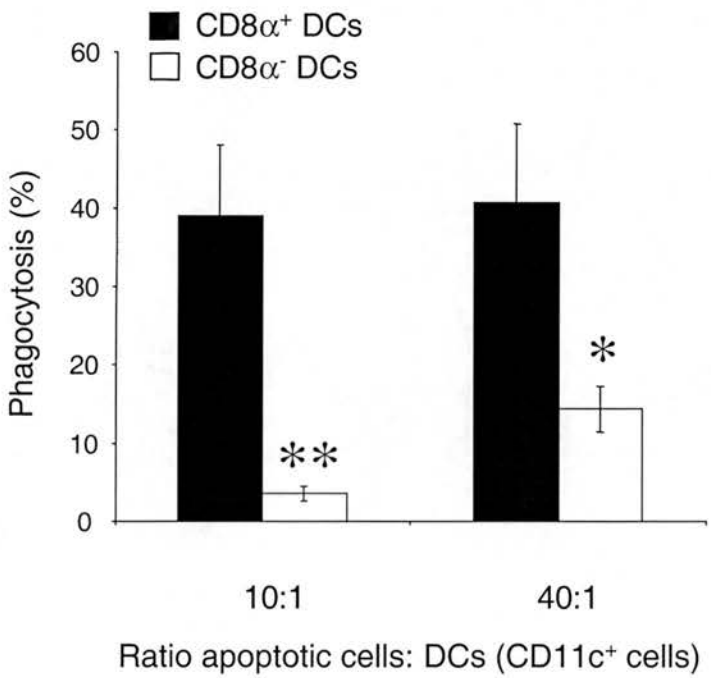


Figure 5.4: Phagocytosis of apoptotic cells by *ex vivo* DCs. Mouse spleen cells were incubated with fluorescently labelled apoptotic human neutrophils (at ratios of 10:1 and 40:1 apoptotic cells: CD11c+ DCs) for 4 hours, and stained for CD11c and CD8α. Phagocytosis was assessed by incorporation of apoptotic cell associated fluorescence into $CD11c^+$ $CD8\alpha^+$ or $CD11c^+$ $CD8\alpha^-$ cells (DCs). Results are expressed as mean percentage phagocytosing cells +/- s.d. for three replicate measurements.

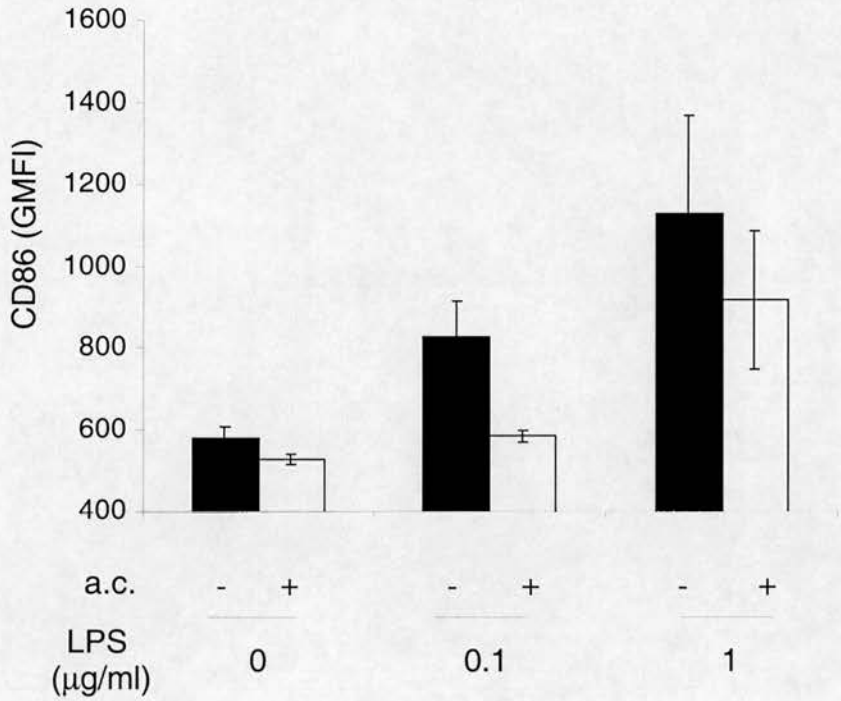


Figure 5.5: Maturation of *ex vivo* DCs following apoptotic cell uptake. Mouse spleen cells were incubated with apoptotic human neutrophils for 2 hours, stimulated with LPS for 24 hours and maturation assessed by surface expression of costimulatory molecule CD86. DCs were gated by CD11c expression and CD86 GMFI measured by FACS. Data are presented as mean GMFI +/- s.d. from three replicate cultures.

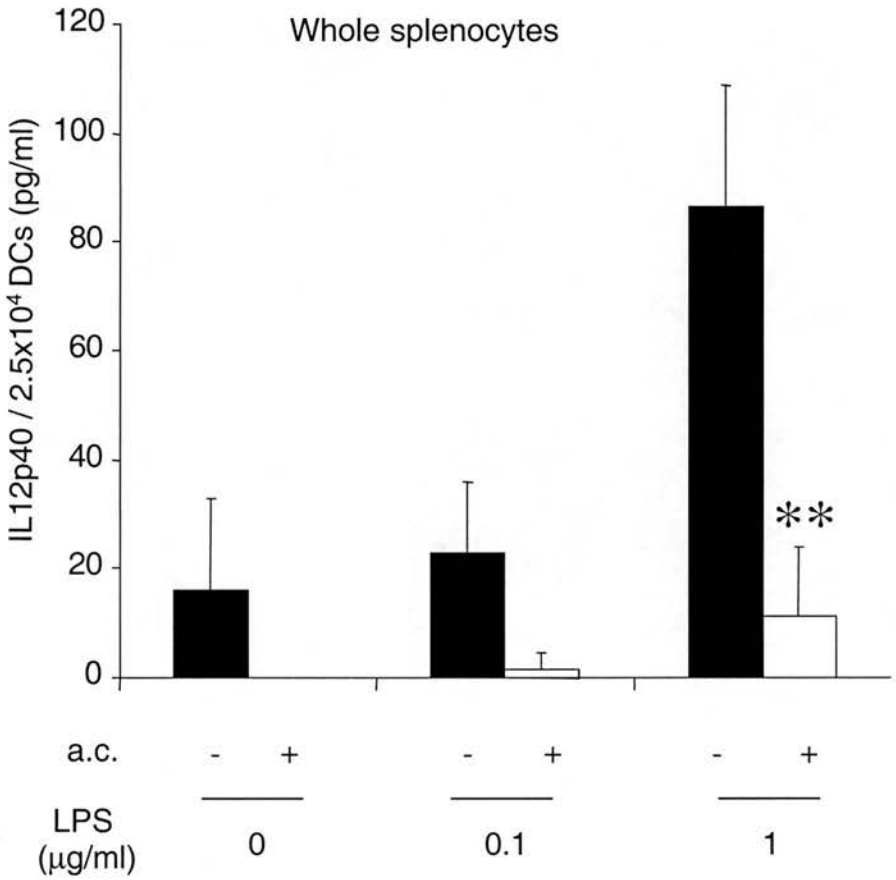


Figure 5.6: IL12 production by splenocyte cultures following apoptotic cell uptake. Mouse spleen cells were incubated with apoptotic human neutrophils for 2 hours, stimulated with LPS for 24 hours, culture supernatants harvested and IL12 measured by ELISA. Data are presented as mean IL12 production/ 2.5 x 104 DCs (CD11c $^{+}$ cells) +/- s.d. from three replicate cultures.

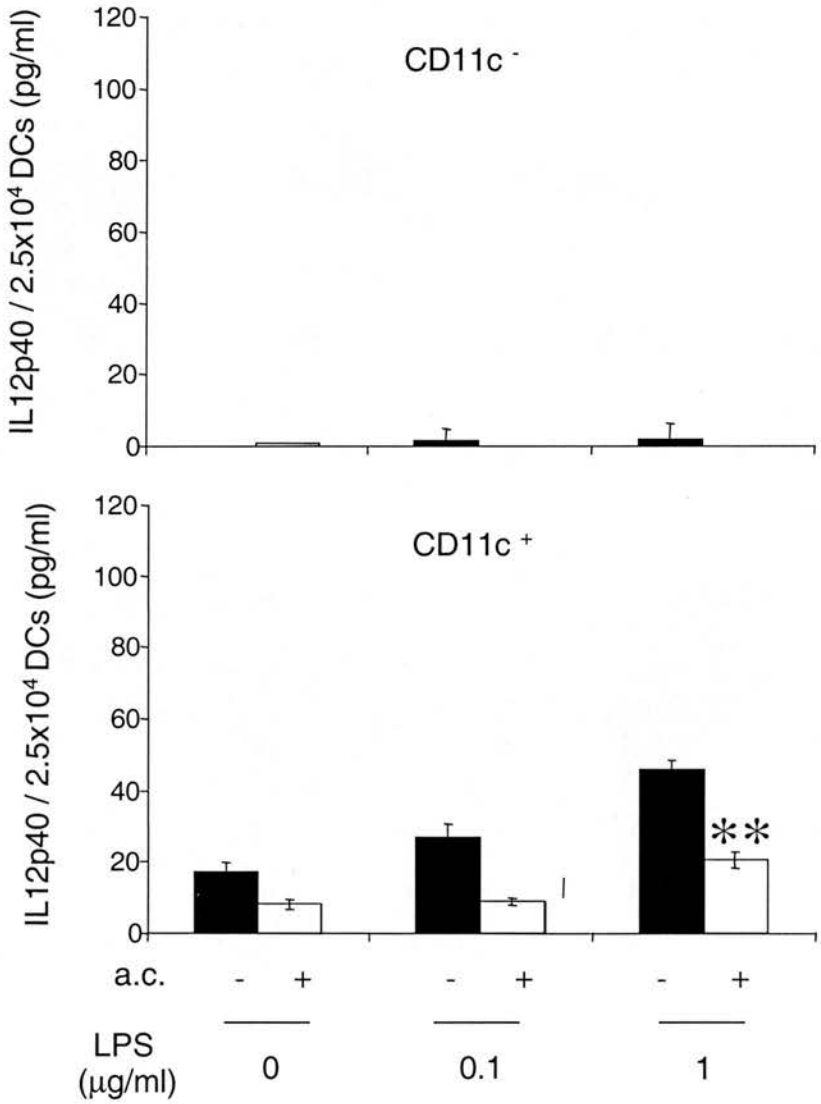


Figure 5.7: IL12 production by purified *ex vivo* DCs following apoptotic cell uptake. *Ex* vivo DCs were purified from mouse spleen cells by postive sorting for CD11c $^{+}$ cells. CD11c $^{+}$ and CD11c $^{-}$ cells were incubated with apoptotic human neutrophils for 2 hours, stimulated with LPS for 24 hours, culture supernatants harvested and IL12 measured by ELISA. Data are presented as mean IL12 production/ 2.5 x 10^4 DCs (CD11c $^{+}$ cells) +/- s.d. from three replicate cultures.

5.2.5 Immunisation of small loads of apoptotic cells *in vivo* is sufficient to establish systemic suppression of DC cytokine production

To test the in vivo effect of apoptotic cell load, mice were immunised either subcutaneously or intravenously with apoptotic cells. These mice were then challenged with endotoxin (80μg/mouse) and systemic production of IL12 assessed at 4 hours, previously shown to be when the peak of DC production of this cytokine occurs. Interestingly, despite using only $8x10^6$ apoptotic cells, in both situations (intravenous and subcutaneous immunisations) a significant inhibition of systemic IL12 (Fig 5.8) occurred, implying that the inhibitory effects of apoptotic cells were spread to bystander leucocytes that were unlikely to have opportunities to interact directly with them. It is possible that some of these systemic effects were mediated by inhibitory cytokines produced by macrophages as well as direct effects on DCs. Notably, although this is not the ideal time point to study TNFα production in response to LPS (the peak being at less than one hour), it did not appear to be modified by apoptotic cells (Fig 5.8).

5.3 Discussion

In this section the ability of apoptotic cells to inhibit freshly isolated murine DCs has been examined. Isolated splenic DCs efficiently internalised apoptotic cells and, as with murine bone marrow derived DCs, apoptotic cells inhibited DC maturation and IL12 production. Furthermore, apoptotic cell administration *in vivo* inhibited systemic IL12 production. Interestingly, as with my bone marrow derived system, human apoptotic cells were able to inhibit murine DCs implying recognition of a common characteristic of apoptotic cells that is conserved across species barriers.

Early systemic IL12 release in response to intravenous endotoxin challenge *in vivo* has been shown derive primarily from CD8α+ dendritic cells (Reis e Sousa et al. 1997; Hochrein et al. 2001) and using this model I have attempted to study

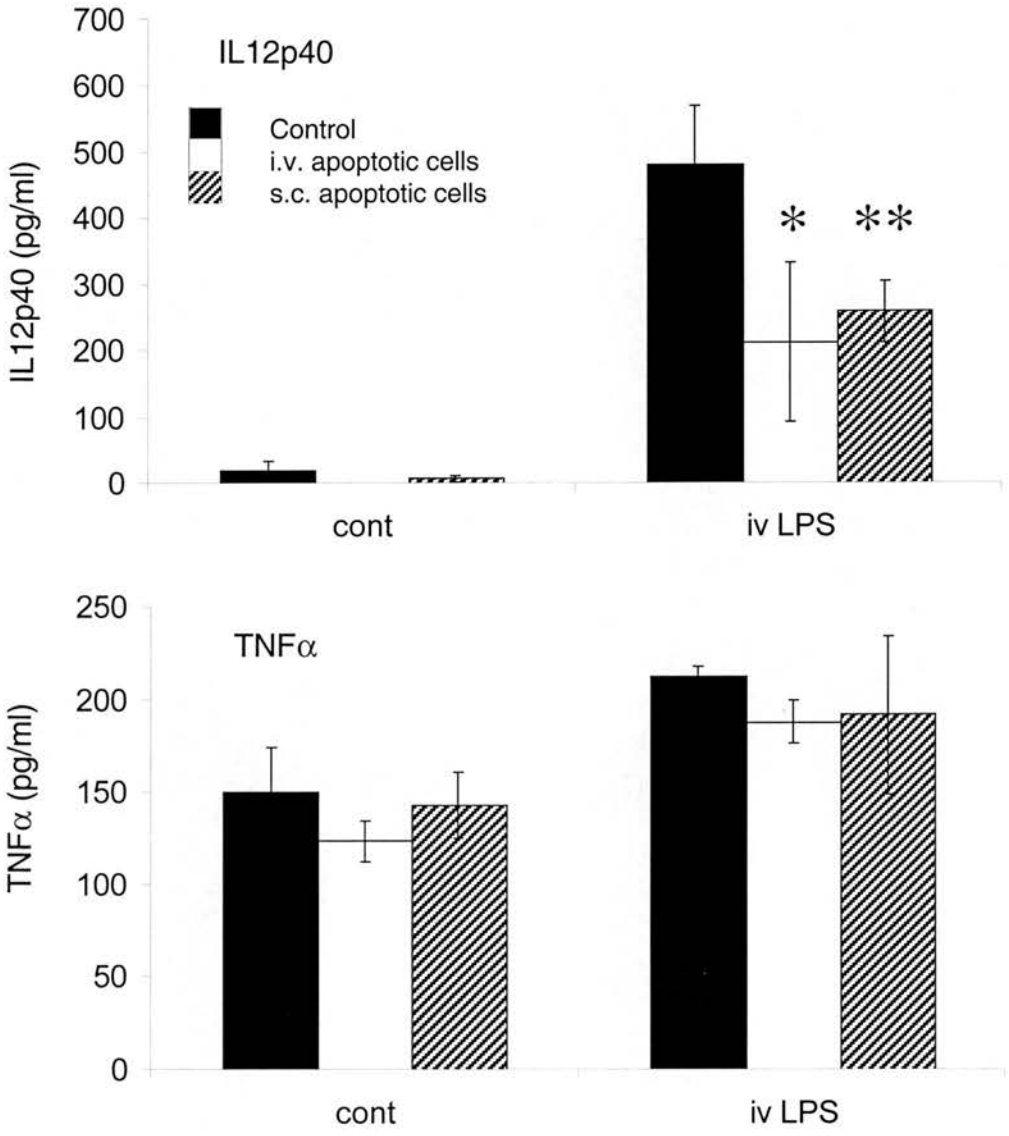


Figure 5.8: Serum cytokine production in mice following injection of apoptotic cells and LPS. Mice were injected with LPS (80μg/mouse intravenously) and apoptotic human neutrophils (8 x 10^6 cells intravenously or subcutaneously). After 4 hours mice were bled under terminal euthanasia serum levels of IL12 and TNF-a measured by ELISA. Co-injection of apoptotic cells reduced serum IL12 levels. Data are presented as mean +/- s.e.m. from 3 to 5 mice. *, ** data are significantly different from mice injected with LPS alone (*$p < 0.05$, $p < 0.01$**, ANOVA).

the effects of apoptotic cells on DC function *in vivo*. However, LPS is not a potent stimulus for IL12p70 but rather produces IL12p40 without induction of the IL12p35 subunit and it was for this reason that it was necessary to study IL12p40 modulation. However, IL12p40 has been shown to be able to form homodimers suggested to block the signalling of the biologically active IL12p70 heterodimeric complexes (Trinchieri 2003). It is possible therefore to envisage that the reduction of IL12p40 might paradoxically increase signalling of IL12p70 and it would be necessary to measure total IL12p70 or a functional readout of its production (such as T cell priming) to ascertain the exact biological effect of apoptotic cell administration. It would be interesting therefore to extend these studies to other stimuli which produce strong Il12p70 signals as well as investigating modulation of adaptive responses and T cell priming.

The observations that CD8α+ DCs express CD36, efficiently internalise apoptotic cells (as demonstrated by our *in vitro* data and by recent publications (Belz et al. 2002b; Iyoda et al. 2002; Schulz et al. 2002a)) and that apoptotic cells modulate the response of this DC subset to LPS raise tantalising possibilities as how this may occur *in vivo*. In my system no IL10 was detected nor were TNFα levels modulated at the time point analysed. Although we cannot exclude modulation of cytokines (especially those produced by macrophages) at an earlier time point than we tested, these data suggest that apoptotic cells may well have direct effects on DCs. However, it would seem likely that the inhibitory effects of apoptotic cells act may also through systemic inhibitory cytokines secreted by macrophages/monocytes or 'switch off' of macrophage proinflammatory mediator release and will be an interesting area of future study (see Chap7).

Conditions inducing large amounts of apoptosis *in vivo*, such as certain infections and UV irradiation, appear to be associated with immunosuppressive effects both locally and systemically (Yoshida et al. 1998). In support of this, three interesting reports have demonstrated the ability of apoptotic cells to induce tolerance. In a murine model of bone marrow transplantation, co-immunisation of apoptotic cells along with the bone marrow permitted

engraftment across MHC barriers (Bittencourt et al. 2001). In addition, another elegant model demonstrated that induction of limited apoptosis in the pancreas of NOD mice protected against autoimmune diabetes by inducing tissue specific tolerance (Hugues et al. 2002) supporting previous results demonstrating a role for limited cell death in inducing tolerance in a model of cross-presentation. Finally, elegant studies by Liu et al have demonstrated that immunisation of mice with small loads of dying cells can indeed induce antigen specific deletion of T cell clones (Liu, K. et al. 2002). These data support a powerful immunoregulatory effect of apoptosis and our data would further suggest that it is mediated, at least in part, by the effects of apoptotic cells on DC activation.

We have shown that *ex vivo* CD8α+DCs preferentially express CD36, an important receptor involved in recognition and internalisation of apoptotic cells and that they efficiently phagocytose dying cells. We postulated that CD8α+DC clear small numbers of apoptotic cells during normal cellular homeostasis *in vivo* and that this might increase during high rates of apoptosis or upon immunisation with dying cells. Indeed, CD8α+DCs did internalise apoptotic cells *in vitro* and examination of the functional sequela of such clearance demonstrated that apoptotic cells could inhibit IL12 production by both *ex vivo* DCs and when injected *in vivo*. Although it is difficult to determine whether this 'modulated' activation may contribute to the functional phenotype of CD8α+DCs *in vivo* the corollary is true; bone marrow derived DCs can be induced to cause T cells to undergo rapid AICD, a characteristic unique to CD8α+DCs, if they contain apoptotic cells (see Chapter 4).

CHAPTER 6: EXAMINATION OF INHIBITORY MECHANISM OF APOPTOTIC CELLS ON DENDRITIC CELLS

6.1 Introduction

Bone marrow-derived DCs and macrophages both arise from myeloid precursors and have many characteristics in common whilst maintaining important differences in responses and effector functions. Macrophages and DCs share a variety of receptors including Toll-like receptors, Fc receptors and scavenger receptors but respond to ligand engagement very differently, modulating effector functions distinct to each cell type (Austyn 1996; Martinez-Pomares et al. 1996). For example, macrophages, specialised in killing and disposal of internalised pathogens, respond to many pathogens with an oxidative burst; in contrast DCs respond to the same stimuli by upregulation of costimulatory molecule expression, important in their prime function as antigen presenting cells. The previous data support evidence showing that DCs bind and ingest apoptotic cells but modulate cytokines differently from macrophages in response to this stimulus (Fadok et al. 1998b; Urban et al. 2001; Stuart et al. 2002). Nevertheless, in both cell types the response is predominantly anti-inflammatory/immunosuppressive and may be linked to common receptor and signalling pathways with differences between these two phagocytic cells being determined by cell type specific 'hard-wiring' to effector functions.

Previous reports emphasise that this phenotypic change of macrophages ingesting apoptotic cells is 'spread' to surrounding cells, through paracrine action of anti-inflammatory cytokine release triggered by the ingestion of apoptotic cells (Voll et al. 1997a; Fadok et al. 1998b). This is in contrast to the response of DCs ingesting apoptotic cells, which, in this current study, did not affect the ability of neighbouring DCs that had not ingested apoptotic cells to mature or stimulate T cells. This would implicate a direct and cell specific effect of apoptotic cell ingestion on DC expression of CD86 and cytokine production, rather than a paracrine effect of secreted anti-inflammatory cytokines. In this

chapter I have investigated these two possible mechanisms of inhibition (i.e. secreted anti-inflammatory cytokines and direct receptor ligation) that could be responsible for the inhibitory effects of apoptotic cells.

6.2 Section 1: Anti-inflammatory cytokines

Studies have concentrated on two major anti-inflammatory cytokines, TGF-β and IL10.

TGF-β

TGF-β1 is a pleiotropic anti-inflammatory cytokine with diverse effects on cell survival, differentiation and proliferation (Shull et al. 1992; Kulkarni et al. 1993; Massague et al. 2000). Systemic and local effects of TGF-β differ; infusion of high levels of systemic TGF-β causes significant immunosuppression whilst local infusion is associated with fibrosis and hypercellularity. TGF-β beta has been shown to alter macrophage phenotype inducing them to adopt an 'alternative' phenotype producing less nitric oxide and driving Th2 responses (Mills et al. 2000) whilst in DCs, it inhibits myeloid DC maturation (Geissmann et al. 1999) and yet is essential for generation/maintenance of Langerhans cells in the periphery which are conspicuously absent from the dermis of *TGF-β-/-* mice (Borkowski et al. 1996) Many cells produce TGF-β, including fibroblast and regulatory T cells, and in tissue, macrophages are an important source of this key cytokine. However, in an interesting recent report (Chen et al. 2001), apoptotic T cells have been shown to produce both active and latent TGF-β localized to intracellular membrane compartments including the mitochondria suggesting that they may actively contribute to TGF-β accumulation in inflamed and remodelling sites as well as in lymphoid tissue. TGF-β exists in a latent form which requires activation before it can signal via a heterodimeric receptor complex composed of type I and type II receptors (Massague 1998). Of

relevance, TGF-β activation can be augmented by TSP, the vitronectin receptor and macrophages themselves (Murphy-Ullrich et al. 1992; Schultz-Cherry et al. 1993). Furthermore, ingestion of apoptotic cells stimulates macrophages to adopt an anti-inflammatory phenotype, inhibiting LPS-induced release of TNFα and upregulating release of TGF-β1 and other anti-inflammatory mediators (Fadok et al. 1998a; Fadok et al. 2000). Neutralisation of TGF-β in the macrophage system almost completely restores the LPS response of apoptotic-cell treated macrophages, implicating this cytokine in the anti-inflammatory effect.

IL10

IL10 is a key anti-inflammatory cytokine with an essential role in development and maintenance of regulatory T cells (Asseman et al. 1999). The important role of IL10 in the control of inflammation is highlighted by the phenotype of *IL10-/-* mice which spontaneously develop an inflammatory colitis (Hara et al. 2001). IL10 inhibits DC maturation and activation and, under certain circumstances DCs themselves can produce IL10 which acting in an autocrine and paracrine manner, is capable of inhibiting DC maturation (Fiorentino et al. 1991). It is likely that lack of these effects on DCs along with an associated failure to produce regulatory T cells, contribute to the autoimmune phenotype that develops spontaneously in mice deficient in this cytokine. In human systems, DCs (Urban et al. 2001) and monocytes (Voll et al. 1997a; Byrne et al. 2002) cocultured with apoptotic cells release IL10 suggesting a possible role for this cytokine in the anti-inflammatory response in this murine system.

6.2.1 Results

Role of TGF-β1

TGF-β1 was found in our DC :apoptotic cell culture supernatants although levels of serum contamination made determining its origin difficult. However, when soluble TGF-β receptor was added to neutralise any active TGF-β

released, DCs' response to LPS was still inhibited after ingesting apoptotic cells (Fig 6.1). In contrast, soluble TGF-β receptor reversed the ability of apoptotic cells to inhibit TNFα production by macrophages in a parallel system confirming that the reagent neutralised TGF-β. Together, these data suggest that paracrine TGF-β does not contribute significantly to the suppressive effects of apoptotic cells on DCs.

Role of IL10

To investigate if changes in IL10 production by DCs ingesting apoptotic cells might contribute to the different phenotypes, intracellular IL10 production and release into the supernatant were studied. IL10 was detectable in DC culture supernatants but was unaffected by interaction with apoptotic cells or the addition of LPS (Fig 6.2). Intracellular IL10 was difficult to detect reliably over background staining and no differences in levels of IL10 staining between ac^+ and ac^- DCs were detectable (Fig 6.2). Furthermore, blockade of functional IL10 by soluble IL10 receptor did not differentially affect co-stimulatory molecule expression in the two DC subpopulations (Fig 6.2).

To further study the role of IL10, bone marrow derived DCs were generated from *IL10-/-* and WT controls. IL10 inhibits DC maturation without affecting the phagocytic capacity of DCs and in support of these observations *IL10-/-* DCs showed neither a difference in the resting phenotype nor a defect in their capacity to phagocytose apoptotic cells (Fig 6.3). On stimulation with LPS *IL10-/-* DCs showed an increased response, expressing higher levels of CD86 and producing more IL12, consistent with reports of autocrine IL10 inhibiting DC maturation (Fig 6.3). However, apoptotic cell ingestion was able to inhibit both CD86 expression (Fig 6.3) and percentage IL12+ve cells (Fig 6.3) in the *IL10-/-* DCs showing that, in our murine system, IL10 did not contribute to the anti-inflammatory properties of apoptotic cells.

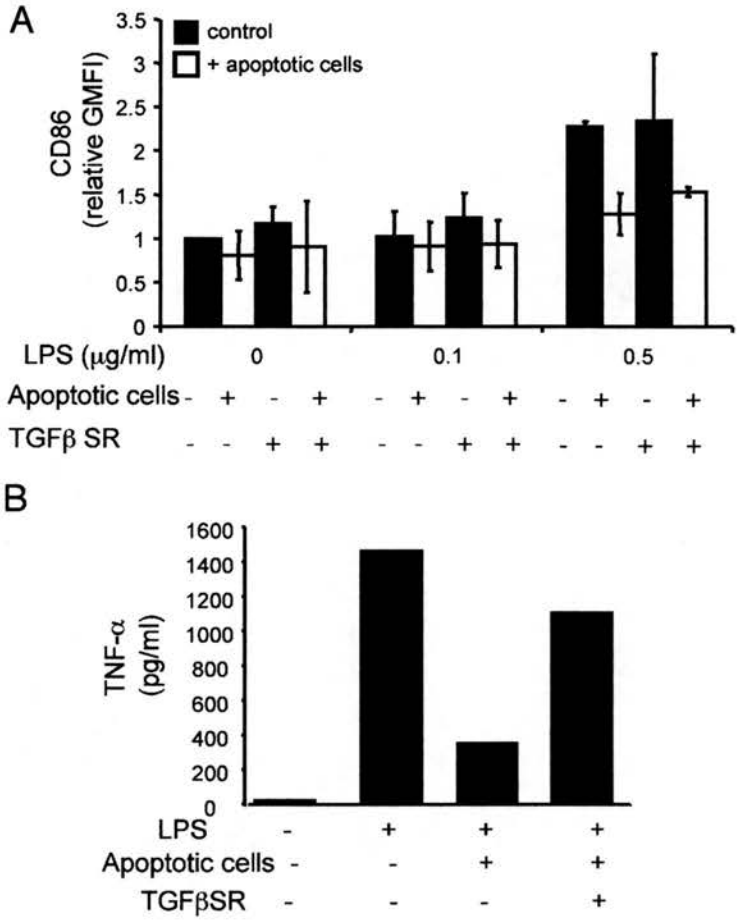


Figure 6.1: Effect of TGFβ neutralisation on DC/ apoptotic cell cocultures. (A) Mouse bone marrow derived DCs were cocultured with apoptotic cells and stimulated with 0.5 μg/ml LPS in the presence or absence of neutralising TGFβSR (0.5 μg/ml). Maturation was assessed by CD86 expression and is expressed as mean GMFI relative to unstimulated cells, +/- s.d. of triplicate measurements. Apoptotic cells continued to inhibit DC maturation despite inhibition of TGF-β signalling. (B) Mouse bone marrow derived macrophages were incubated with apoptotic cells and/ or LPS and culture supernatant TNF-α levels measured after 24 hours. TGFβSR inhibited the effect of apoptotic cells on TNF-α production confirming a role for TGF-β1 in macrophage reprogramming (Figure B courtesy of Mark Lucas).

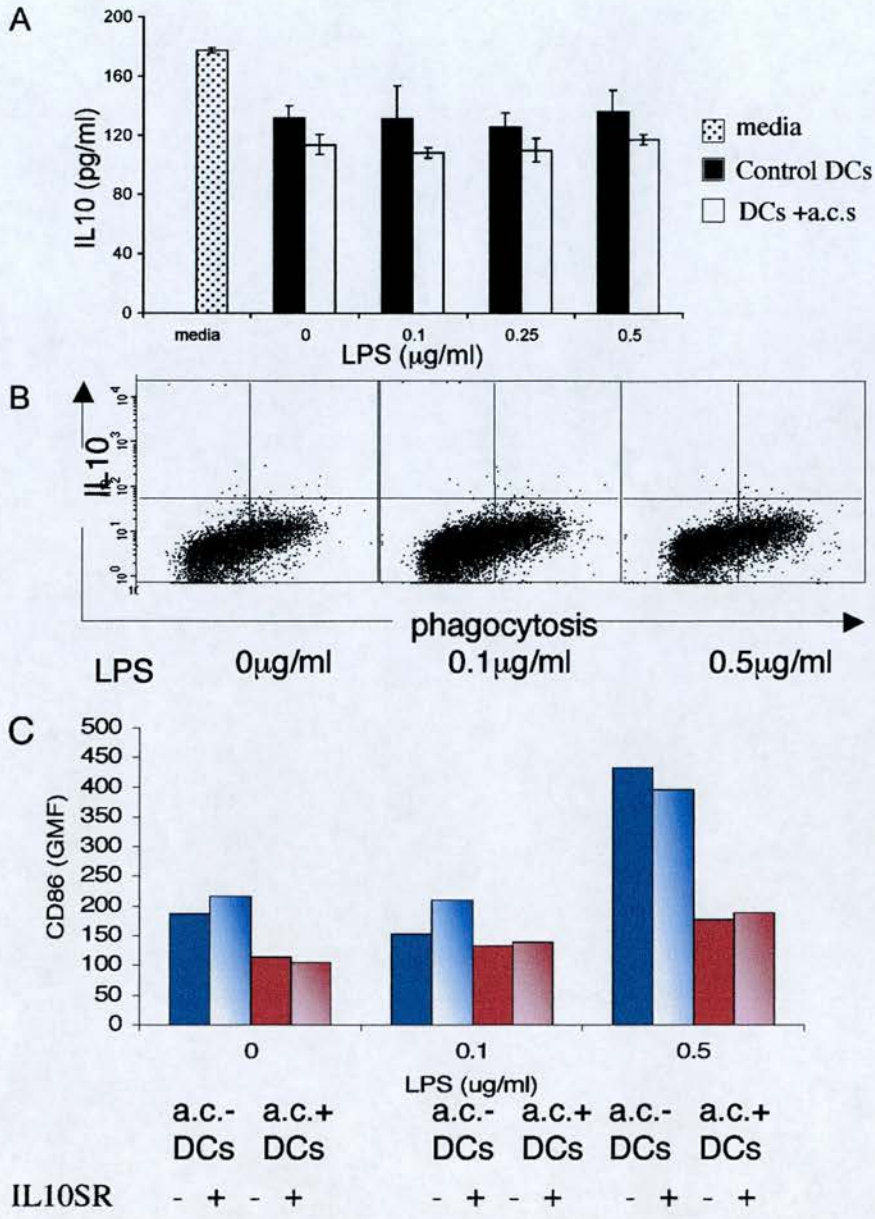


Figure 6.2: Role of IL10 in apoptotic cell modulation of DCs. **A:** Mouse bone marrow derived DCs were incubated with apoptotic cells, stimulated with LPS for 24 hours and IL10 measured in culture supernatants. Data are expressed as mean +/- s.d. for triplicate measurements. Also shown is IL10 levels in culture medium. **B:** DCs were incubated with fluorescent apoptotic cells, stimulated with LPS and stained for intracellular IL10 after 4 hours. DCs were gated from apoptotic cells by size, and are plotted as phagocytosis (apoptotic cell associated fluorescence) against IL10. Quadrant markers are set on isotype stained/ unstimulated DCs. **C:** DCs were incubated with apoptotic cells and stimulated with LPS in the presence or absence of IL10 soluble receptor (IL10SR used at 2μg/ml) and maturation assessed by surface levels of CD86.

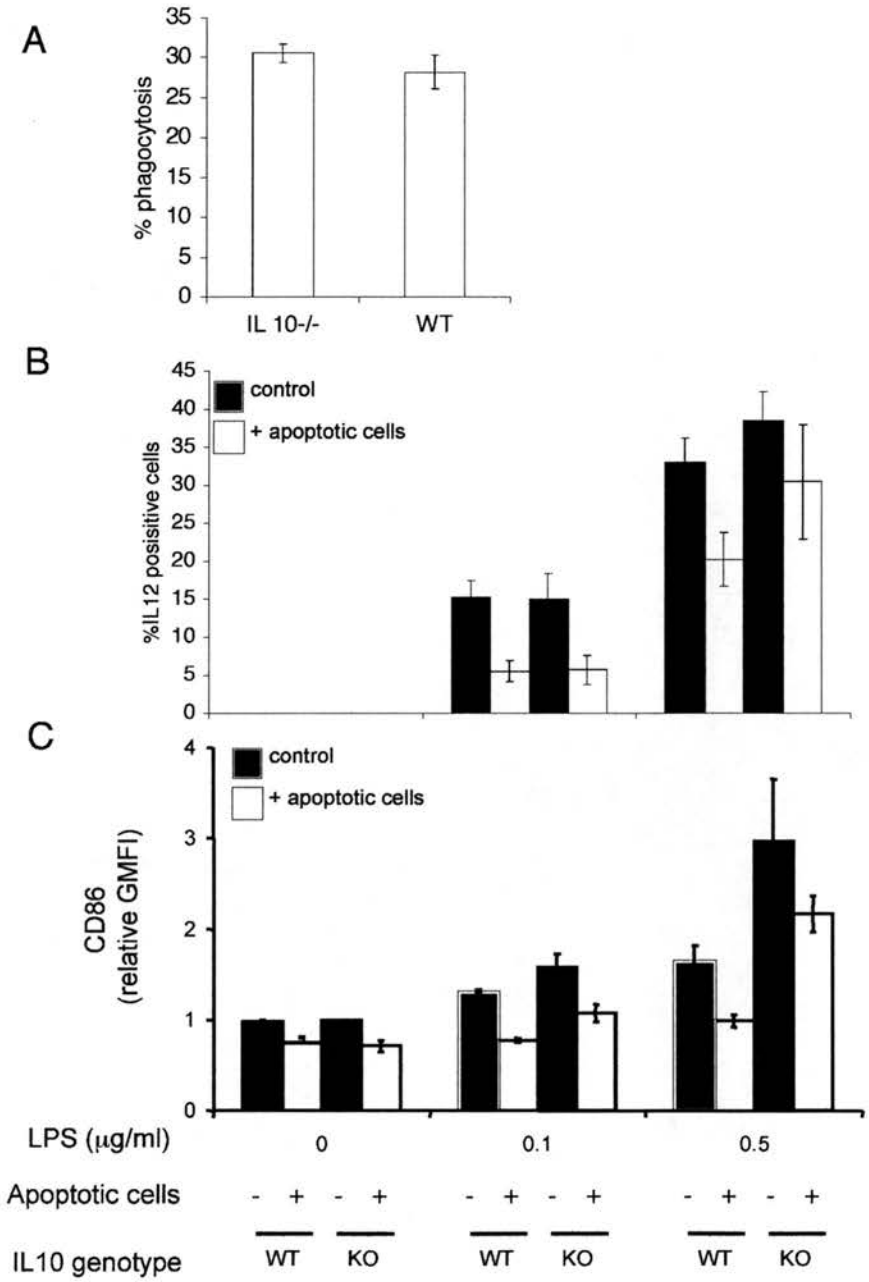
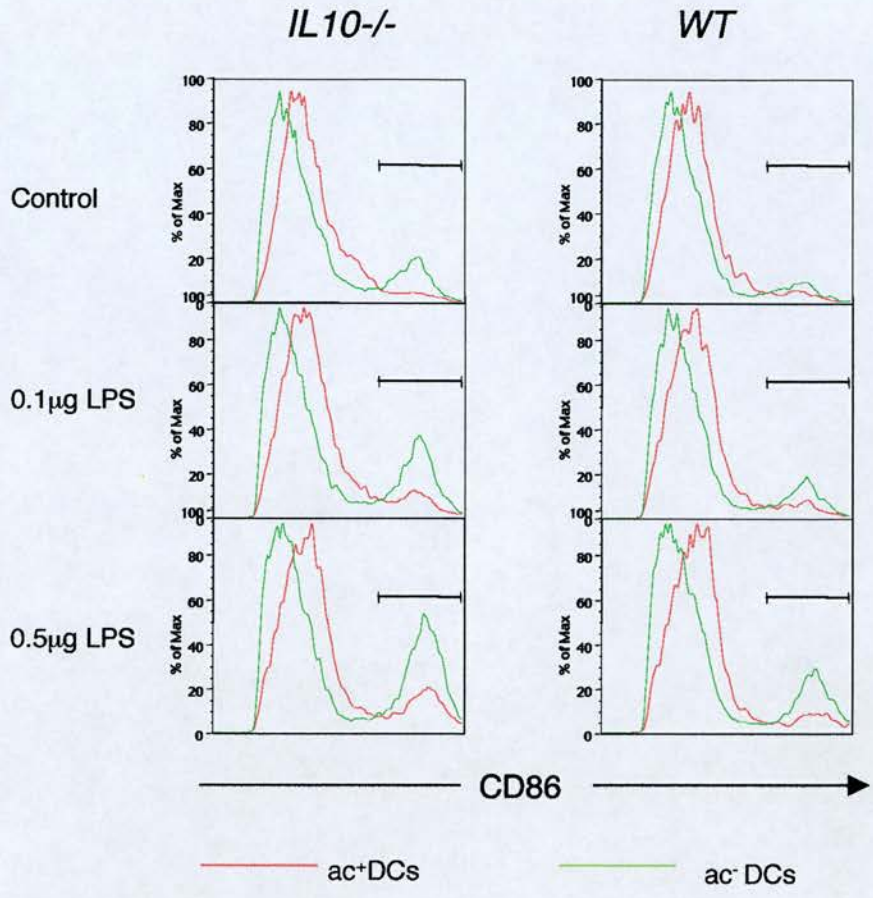


Figure 6.3: Apoptotic cell phagocytosis and response by IL10 $^{-/-}$ DCs. Bone marrow derived DCs were prepared from IL10 $^{-/-}$ and control mice, incubated with apoptotic human neutrophils and stimulated with LPS. **A:** Phagocytosis of fluorescent apoptotic cells was measured after 4 hours (2:1 ac: DC ratio) by FACS, and is expressed as mean percentage phagocytic DCs (CD11c+ cells)+/- s.d. for triplicate samples.**B, C:** DC maturity was assessed after 24 hours by intracellular cytokine staining or surface CD86 expression.

D



E

		IL10-/-	WT
		% CD86 high	% CD86 high
Control	ac+DCs	6.41	5.84
	ac- DCs	11.5	8.01
0.1μg LPS	ac+DCs	8.28	5.48
	ac- DCs	16.3	9.45
0.5μg LPS	ac+DCs	13.5	8.12
	ac- DCs	24.2	15.9

Figure 6.3: Apoptotic cell phagocytosis and response by IL10 $^{-/-}$ DCs. Bone marrow derived DCs were prepared from IL10 $^{-/-}$ and control mice, incubated with apoptotic human neutrophils and stimulated with LPS. **D:** Representative histograms of expression of CD86 in ac+ and ac- DCs in WT and *IL10-/-* DCs. **E**: Table showing percentage CD86 high in ac+ and ac-DCs.

6.2.2 Discussion

The initial observations that only those DCs that had phagocytosed were inhibited, whilst bystander DCs remained unaffected suggested that a soluble mediator was unlikely to be responsible for these inhibitory effects. However, data published during the course of this work, demonstrated release of IL10 by human DCs upon culture with apoptotic cells (Urban et al. 2001) suggesting a possible role for this cytokine. Our initial strategy of neutralisation of two candidate cytokines, TGF-β and IL10, failed to reverse inhibitory effects and further mitigated against a role for secreted factors in the anti-inflammatory actions. Interestingly, neutralisation of IL10 was reported to be unable to reverse apoptotic cell inhibition of DC maturation by Urban in the human DCs. We then elected to perform definitive experiments using DCs generated from *IL10-/-* bone marrow. These DCs phagocytosed normally and were also inhibited by apoptotic cell ingestion eliminating the possibility that production of this cytokine was responsible for the inhibitory phenomenon.

6.3 Section 2: Phagocytic receptor ligation

The preferential inhibition of IL-12 is a common consequence of ligation of a large number of receptors on macrophages including Fc and complement receptors (Mosser et al. 1999) and receptors used by viruses such as measles (Fugier-Vivier et al. 1997; Sutterwala et al. 1999) and vaccinia (Engelmayer et al. 1999). In contrast, only specific ligands will induce production of IL10 or down regulation of TNFα demonstrating both conserved and divergent signalling pathways and responses linked to phagocytosis (Sutterwala et al. 1998; Mosser et al. 1999; Gerber et al. 2001a; Gerber et al. 2001b; Grazia Cappiello et al. 2001). Therefore, in macrophages, the receptor used determines the type of response generated; for instance IL10 production along with inhibition of IL12 appears to be a feature of FcγR1 ligation but not complement receptor mediated phagocytosis. Similarly, ligation of both the vitronectin receptor (Voll et al, 1997 ; Freire-de-Lima et al, 2000) and PsR (Fadok et al. 2000) induce production of TGF-β from macrophages. It has been suggested that receptor ligation may act alone or in synergy with autocrine inhibitory cytokine production to cause the inhibitory effects after phagocytosis by macrophages (Mosser et al. 1999) and this may also be the case after internalisation of apoptotic cells. Furthermore, it is possible that the different outcome of macrophage and DC phagocytosis of dying cells may be determined by the distinct recognition mechanisms employed for their clearance.

CD36

CD36 is a heavily glycosylated integral type II membrane protein with multiple ligands including apoptotic cells, thrombospondin (Silverstein et al. 1992), long chain fatty acid, modified lipids, malaria parasitized erythrocytes, sickled erythrocytes and rod outer segments in the eye (Fig 6.4) (Febbraio et al. 2001). CD36 is found localised in lipid rich caveoli where it is thought to have multiple functions including signalling, regulation of caveolin function and uptake of modified lipoproteins and long chain fatty acids. Although a detailed

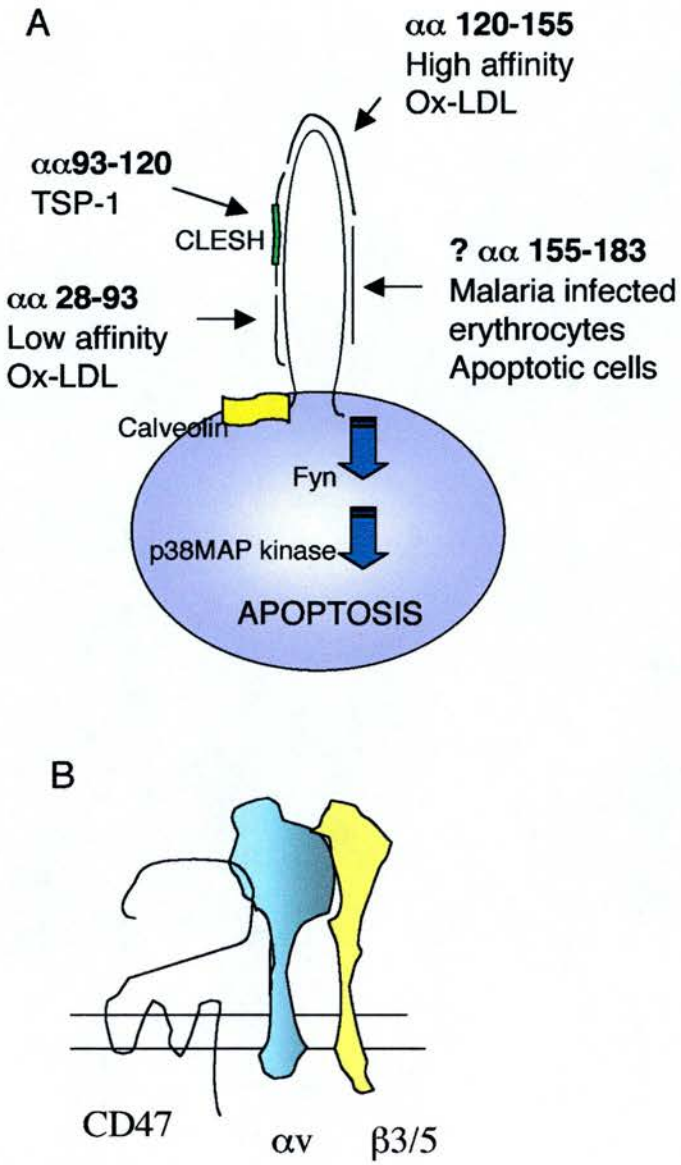


Figure 6.4: Putative DC receptors for apoptotic cells. A: CD36 CD36 is a multiligand receptor able to bind malaria parasitized erythrocytes, ox LDL and thrombospondin. Cartoon shows the proposed membrane organisation of CD36, with identified ligand binding sites marked with primary sequence position (amino acid, αα). **B:** αv integrins αvβ3 and αvβ5 are implicated in apoptotic cell recognition and may act in conjunction with associated proteins such as CD47.

discussion of all its functions is beyond the scope of this thesis it is important to emphasise its role in lipid handling and the aetiology of atherogenesis (Febbraio et al. 2001).

CD36 is involved in apoptotic cell uptake by both macrophages (Savill et al. 1992; Fadok et al. 1998c) and DCs (Albert et al. 1998a) as well as other non-professional phagocytes such as retinal pigment epithelial cells removing rod outer segments in the eye (Finnemann et al. 2001). In many cell types CD36 is thought to co-operate with integrins αvβ5 and αvβ3 (VnR) to mediate apoptotic cell engulfment. Furthermore, the discovery that a Drosophila homologue of CD36 (croquemort) was important for clearance of dying cells in the fly, has demonstrated a teleologically conserved function for this molecule and underscored its importance (Franc et al. 1996; Franc et al. 1999). Antibody blockade studies in human dendritic cell systems have implicated CD36 in phagocytosis of apoptotic cells. The exact domains of CD36 that bind apoptotic cells are thought to be distinct from binding sites for oxLDL (which binds αα 120-155) and TSP-1 (αα 93-120) but antibody studies have demonstrated that they share their binding domain with malaria parasitized erythrocytes (Fig 6.4). However, it is controversial whether CD36 on macrophage and RPE (Ryeom et al. 1996) (and by inference DC) binds directly to the apoptotic cell and it has recently been demonstrated that ligation of CD36 increases internalisation even when it is unable to bind directly to the target cell suggesting that it has a signalling role as well as a role as a receptor (Finnemann and Silverstein. 2001).

Interestingly, some of the inhibitory effects of apoptotic cells can be recapitulated in human DCs by antibody ligation or by malaria-parasitized erythrocytes (Urban et al. 2001). In their human system, Urban demonstrated selective inhibition of IL12 production and no modulation of TNFα following antibody ligation of CD36 and LPS stimulation of DCs, similar to the results demonstrated in our murine system. Mutations in human CD36 are found commonly in certain areas of Africa and are associated with different susceptibility to cerebral malaria (Aitman et al. 2000) supporting a possible role

for CD36 in modulating the immune response in this infection (Urban et al. 1999). Taken together, these observations suggest that the malaria-parasitized red cells and apoptotic-cells may bind a common site in CD36, which in turn, may be important for consequent anti-inflammatory signalling. We therefore took advantage of mice rendered deficient in this receptor to study the role in both phagocytosis and response of DCs to apoptotic cells.

αvβ3/5 (vitronectin receptor)

Integrins are cell surface receptors composed of an α and β subunit, which heterodimerize and through combinatorial variation create up to 20 receptors with varying specificities. They are important in cell binding to matrix, cell:cell interactions and migration. Non-inflammatory macrophages use αvβ3 integrin (Fig 6.4) for internalisation of apoptotic cells and, although recent work has demonstrated the presence of αvβ5 on these cells, it does not appear to be active for phagocytosis unless PKC activation by PMA has occurred (Finnemann et al. 1997). In contrast retinal pigment epithelial cells do not use αvβ3 but express and utilise αvβ5 for phagocytosis, which is unaffected by activation with PMA. Human DC phagocytosis of apoptotic cells is thought to proceed after ligation of αvβ5 integrin which is linked to an unidentified tyrosine kinase shown to recruit p130cas, CrkII and DOCK180 resulting in Rac1 activation and actin polymerisation (Albert et al. 2000; West et al. 2000; Giles et al. 2001). Although αvβ5 has been implicated in DC phagocytosis of apoptotic cells in human systems (Albert et al. 1998a) other groups have suggested that DCs may also use β3 integrins (Rubartelli et al. 1997) and it remains unresolved whether DCs may in fact be capable of utilising both integrins interchangeably depending on PKC activation. For these reasons, DCs generated from mice null for both β3 and β5 integrins were used to study their role in the anti-inflammatory response.

6.3.1 Results

Role of CD36

DCs were generated from wild type mice and mice rendered deficient in CD36 that had been screened by PCR to confirm the absence of CD36. First I wished to determine if our WT DCs expressed a functional CD36 receptor at the cell surface but at the time of these experiments an anti-murine CD36 antibody was not available. However, CD36 is considered the principal macrophage receptor for OxLDL and we chose to examine this function in our WT and *CD36-/-* DCs as a surrogate for CD36 receptor expression . WT DCs ingest OxLDL, but in contrast to ingestion of acetylated LDL (thought to be predominantly SRA mediated), DCs exhibit either high or low capacity for OxLDL uptake possibly representing varying levels of maturity in these murine cultures. Interestingly, DCs from *CD36 -/-* mice internalised lipid with similar efficiency to their WT controls probably representing utilisation of additional scavenger receptors (Fig 6.5) able to mediate uptake. However, OxLDL can induce apoptosis a phenomenon that has been shown to be dependent on CD36 ligation. *CD36-/-* DCs were resistant to apoptosis when cultured in OxLDL supplemented media for 4 days (Fig 6.5) whilst WT DCs died in OxLDL rich media. These data demonstrate that *CD36-/-*DCs do indeed lack a functional receptor and confirmed previous reports from human monocyte systems, that CD36 is required for OxLDL mediated cell death (Wintergerst et al. 2000).

The role of this receptor in binding and internalisation of apoptotic cells by DC was also assessed. At 4°C DC binding to apoptotic cells was partially serum dependant, approximately doubling in the presence of serum components in the WT DCs (Fig 6.6). Interestingly, *CD36-/-* and WT DCs bound apoptotic cells equally in the absence of serum but the *CD36-/-* DCs were defective in apoptotic cell binding in the presence of serum (Fig 6.6) compared to WT controls. Thus CD36 binding of apoptotic cells requires a serum component and accounts for approximately 50% of serum dependent binding of DCs to apoptotic cells. Phagocytosis of apoptotic cells at 37°C was normal in the *CD36-/-* DCs as

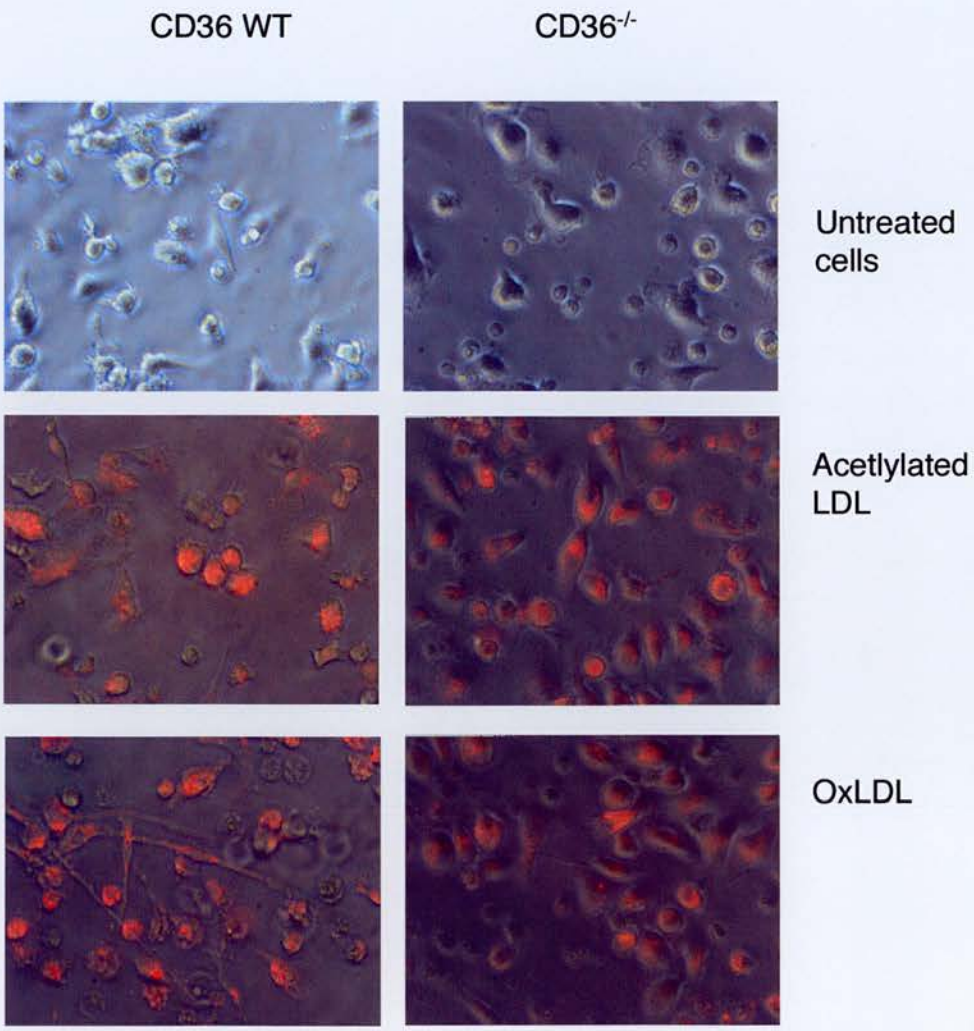


Figure 6.5: CD36 is required for oxidised LDL induced apoptosis of DCs. Bone marrow derived DCs were prepared from CD36 $^{-/-}$ and control strain matched (C57Bl/6) mice, incubated with fluorescent acetylated or oxidised LDL(10μg/ml) for 4 days, and viewed by phase contrasts and fluorescent microscopy (x 40 objective). Images show untreated DCs (phase contrast) and lipid treated cells (phase contrast/ fluorescence composites). OxLDL but not control acetylated LDL induces DC apoptosis in wild type but not CD36$^{-/-}$ cells.

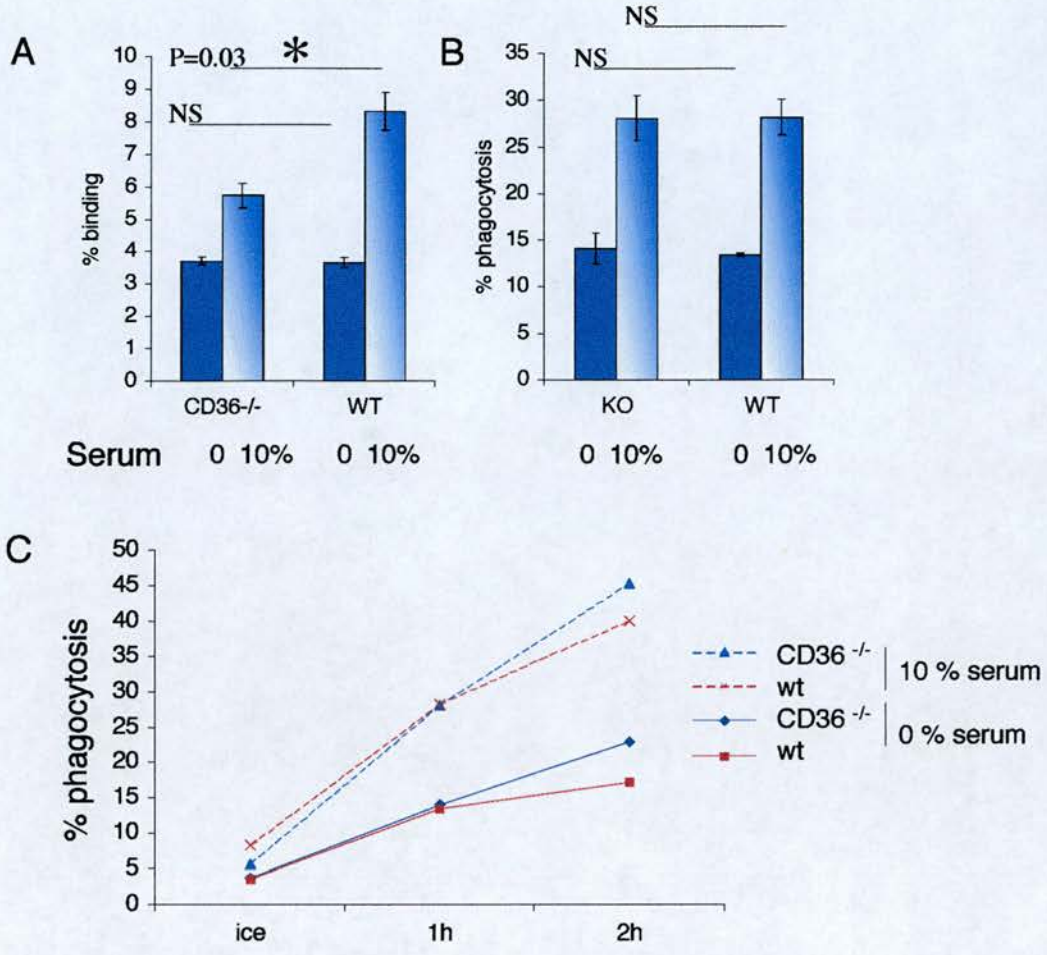


Figure 6.6: CD36 is not required for phagocytosis of apoptotic cells by DCs. Bone marrow derived DCs were prepared from CD36 $^{-/-}$ and control strain matched (C57Bl/6) mice, incubated with fluorescent apoptotic cells and phagocytosis measured by FACS. **A:** DCs were incubated with apoptotic cells with or without serum on ice to prevent phagocytosis and measure tight binding to DCs. **B:** DCs and apoptotic cells incubated together at 37° C for 1 hour to measure phagocytosis. **C:** time course of apoptotic cell phagocytosis. All data are presented as mean percentage phagocytic/ interacting cells +/- s.d. for triplicate measurements.

compared to WT DCs (Fig 6.6). Thus, at higher temperatures permissive for cytoskeletal rearrangement DCs probably recruit additional binding molecules for utilisation in the phagocytic process demonstrating possible 'redundancy' of apoptotic cell recognition.

Finally, upon subsequent challenge with LPS, ac+DCs from both *CD36-/-* and WT mice demonstrated lower CD86 expression (Fig 6.7) and decreased numbers of IL12+cells (Fig 6.7). Interestingly, *CD36-/-* DCs responded to LPS more vigorously than WT controls suggesting that CD36 may indeed provide an inhibitory signal. Although the identity of the ligand is unknown it does not appear to be exogenously added apoptotic cells and I would suggest lipid or TSP contained in serum as possible candidates. Thus, although in human systems ligation of CD36 by antibody may be sufficient to inhibit DC maturation, it does not appear to be necessary for the inhibitory effects of apoptotic cells on mouse DCs.

Role of β3/5 integrins

The vitronectin receptors have been implicated in phagocytosis of apoptotic cells by both macrophages and DCs. I first wanted to establish if our bone marrow derived DCs demonstrated a requirement for integrins to internalise apoptotic cells. Few antibodies are available for murine integrins and we chose instead to use peptides GRGDSP, GRGDTP (specificly inhibits binding to collagen, fibronectin and vitronectin) and the irrelevant control peptide, GRADSP to study the role of integrin-dependent internalisation. DC:apoptotic cell interactions were not affected by the presence of these peptides (Fig 6.8) at 0.5mg/ml although EDTA (used as a positive control) significantly decreased DC phagocytosis confirming previous reports for a dependency on cations for DC internalisation (Fig 6.8).

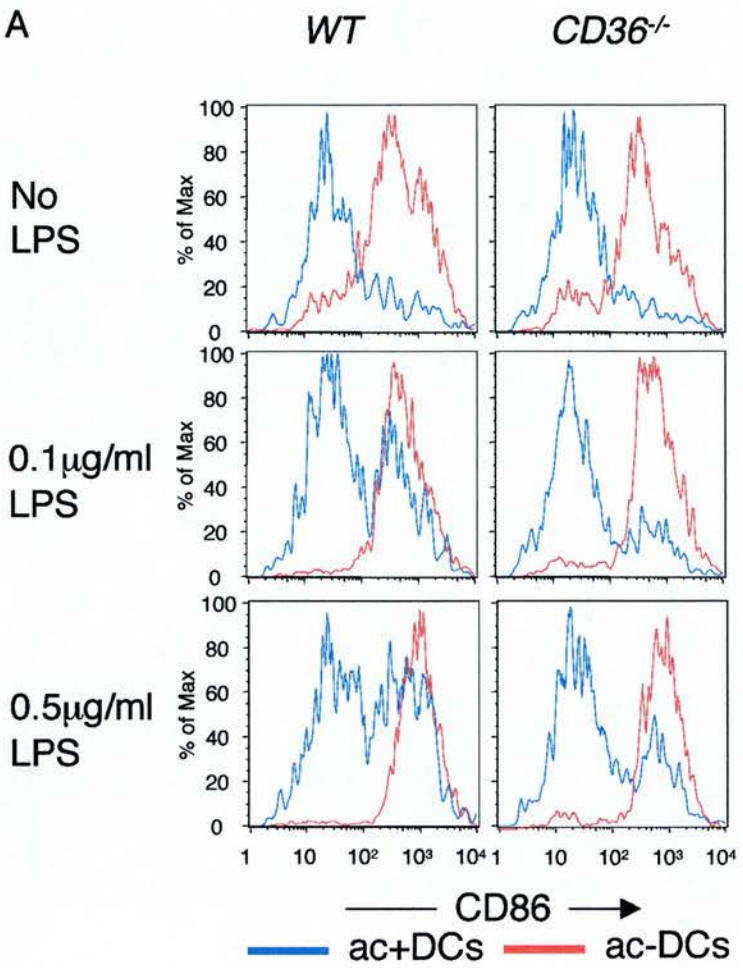


Figure 6.7: CD36 is not required for DC response to apoptotic cells. A: Bone marrow derived DCs were prepared from CD36 $^{-/-}$ and control strain matched (C57Bl/6) mice, incubated with fluorescent apoptotic cells and stimulated with LPS. Cell surface expression of CD86 was analysed by FACS, DCs identified by size and CD11c expression. Data show CD86 expression on DCs separated into those that had ingested apoptotic cells (ac+) from bystander DCs (ac-) by incorporation of apoptotic cell associated fluorescence.

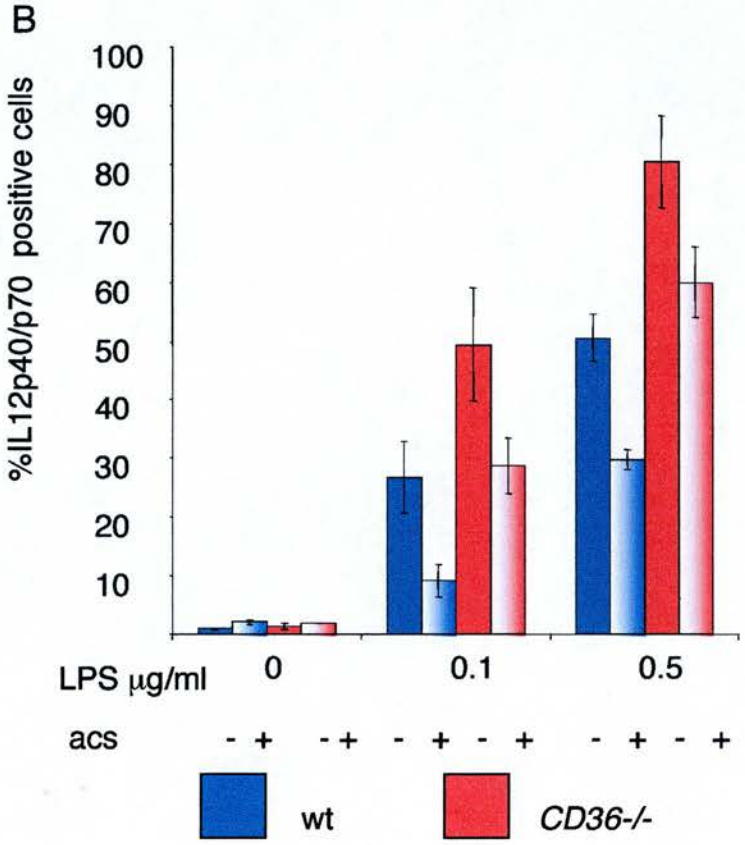


Figure 6.7: CD36 is not required for DC response to apoptotic cells. B: DCs from CD36 $^{-/-}$ and control mice were incubated with apoptotic cells and stimulated with LPS. IL12 producing DCs were detected by intracellular staining, and analysed by FACS (DCs identified by size and CD11c expression). Data show percentage of IL12 producing DCs in cultures with or without apoptotic cells and are expressed as mean percentage +/- s.d. for triplicate measurements. CD36-/- DCs appear to be hypersensitive to LPS.

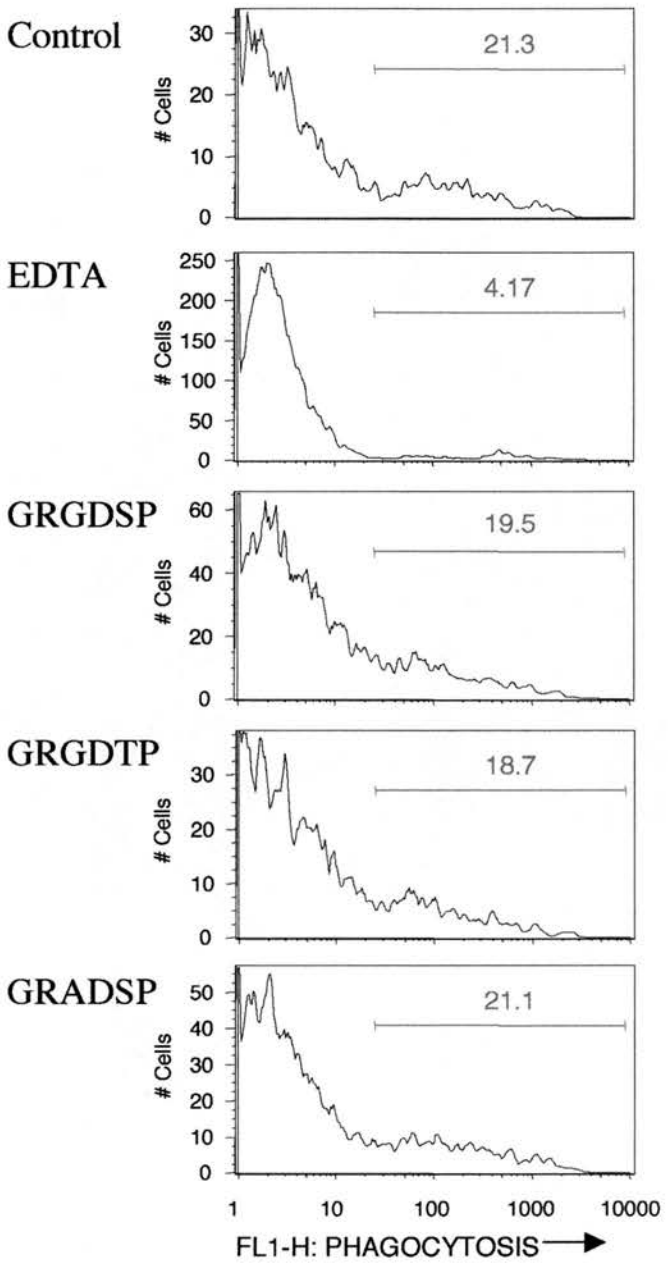


Figure 6.8: Phagocytosis of apoptotic cells by DCs is not blocked by inhibitors of integrin-ligand binding. Mouse bone marrow derived DCs were incubated with fluorescent apoptotic cells in the presence of EDTA (20 mM) or peptide inhibitors of integrin-ligand binding (0.5mg/ml) for 1 hour, and phagocytosis measured by FACS. Profiles are apoptotic cell fluorescence associated with DCs (CD11c+ cells). Numbers show percentage phagocytic cells.

However, these results were at odds with published data on DC phagocytosis using antibody blockade, the results of which heavily implicate integrin β5 and β3 in phagocytosis. We chose therefore to take advantage of mice generated with double targeted deletions for both β3 and β5 integrins (Reynolds et al. 2002). *β5/3-/-* DCs developed normally in culture and appeared to have similar surface phenotype to their WT equivalent. Firstly, we established that DCs generated from our cultures had a functional vitronectin receptor. As antibodies to murine β5 are not available we chose to study an integrin dependant function; binding and spreading on vitronectin coated plates was marked in WT DCs whilst *β5/3-/-* DCs failed to spread on this matrix indicating an absence of a functional receptor (Fig 6.9). WT and *β5/3-/-* deficient DCs bound equally to control polylysine plates (data not shown). To assess other integrins, DCs were also plated onto laminin (which binds α1β1, α2β1, α3β1, α6β, α7β1, α9β1 and α 6β4) and fibronectin (which binds αv and β1 integrin families). Interestingly, slight morphological differences in binding to fibronectin between WT and *β5/3-/-* were seen whilst neither WT nor *β5/3-/-* DCs bound laminin (Fig 6.9). Thus our WT bone marrow derived DCs, unlike our *β5/3-/-* DCs, express functional vitronectin receptors allowing them to adhere and spread on the appropriate matrix.

DCs from these mice were then used to determine the role of these integrins in both phagocytosis and inhibitory signalling to apoptotic cells. The *β5/3-/-* did not appear to demonstrate a defect in apoptotic cell binding at 4°C as compared to WT DCs nor was there a deficiency in their phagocytosis at 37°C (Fig 6.10). Furthermore, the ablility of apoptotic cells to inhibit DC maturation (Fig 6.11) was retained in the *β5/3-/-* DCs mitigating against an absolute requirement for ligation of these receptors in this phenomenon.

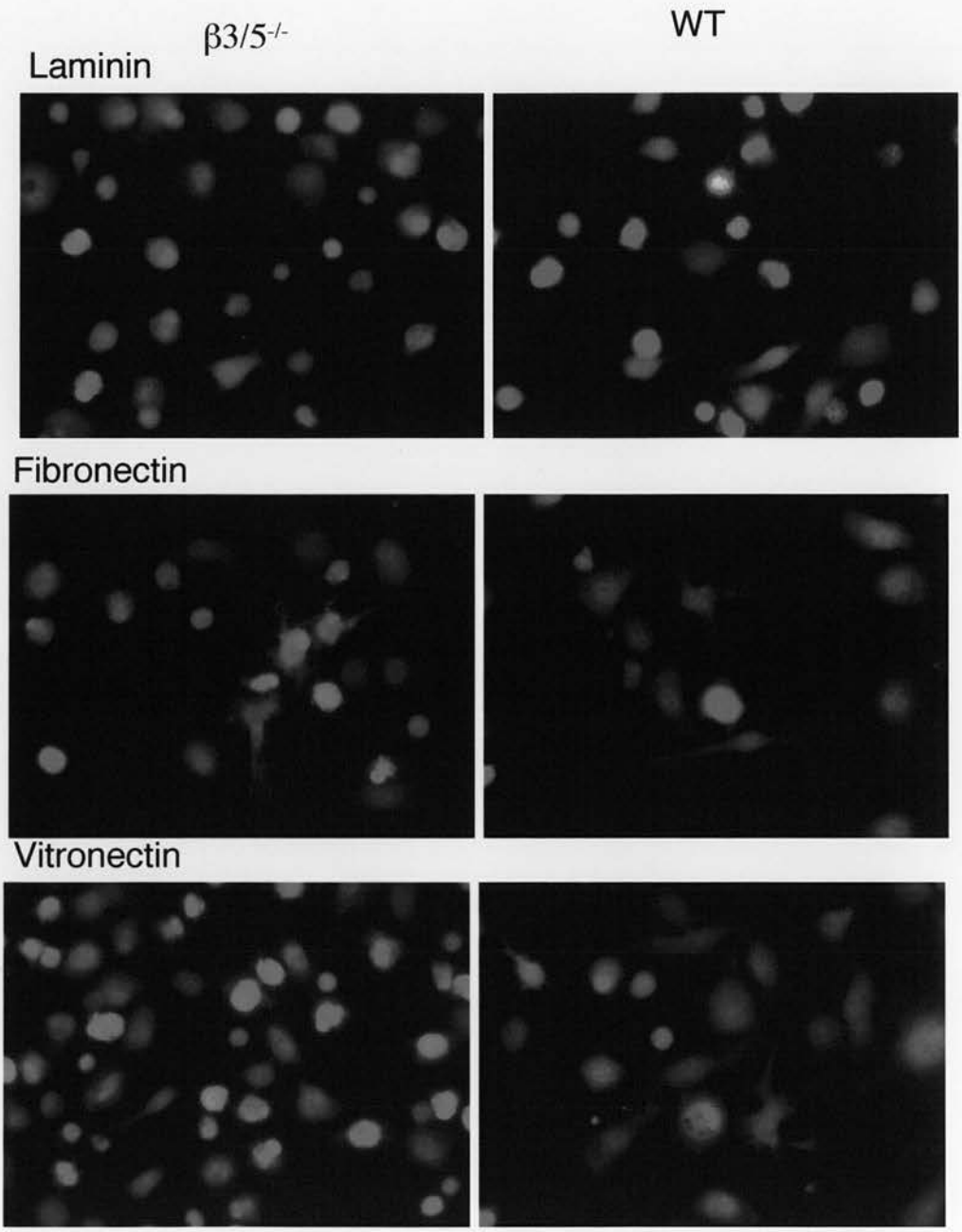


Figure 6.9: Role of β3 and β5 integrins in DC attachment and spreading on matrix. Mouse bone marrow derived DCs from wild type or integrin $\beta3^{-/-}$ $\beta5^{-/-}$ mice were labelled with cell tracker dye (CMFDA), cultured on coverslips coated with matrix components for 1 hour, and visualised by fluorescent microscopy. Pictures show representative fields of cells viewed with x 40 objective. Control cells clearly spread more when plated on vitronectin.

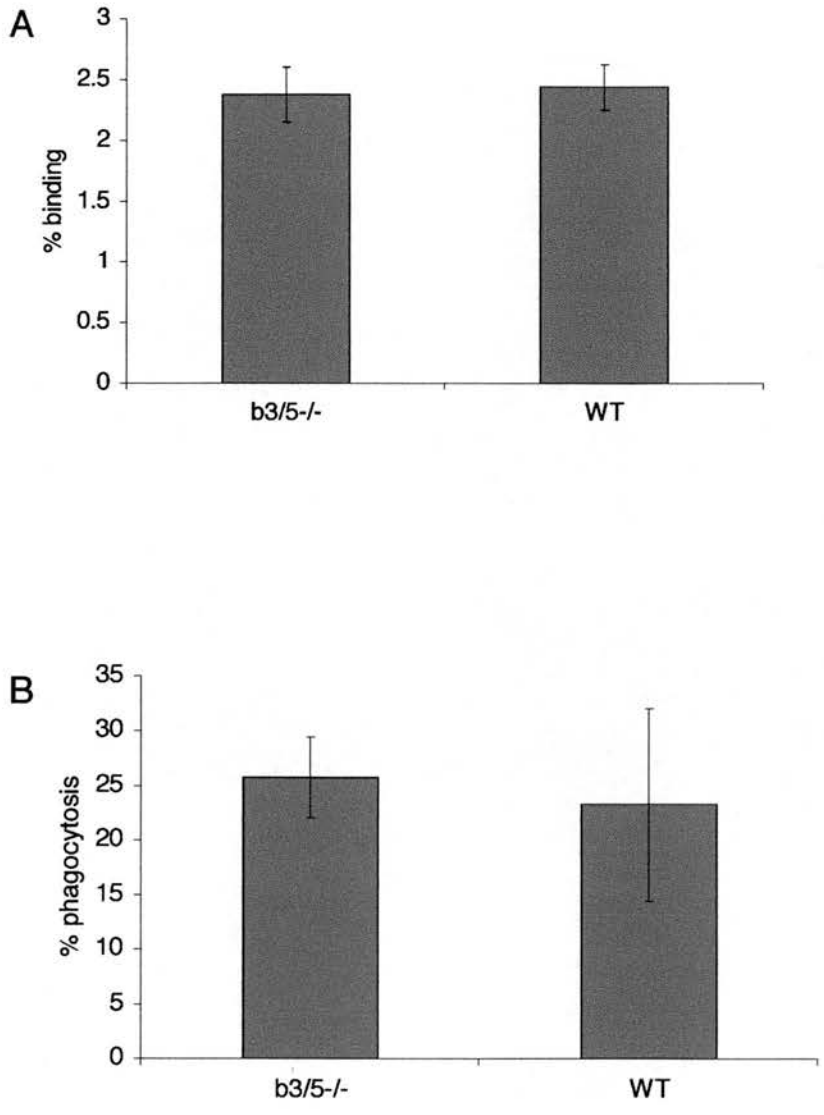


Figure 6.10: Role of β3 and β5 integrins in apoptotic cell binding and phagocytosis by DCs. Mouse bone marrow derived DCs from wild type or integrin $\beta3^{-/-}$ $\beta5^{-/-}$ mice were incubated with fluorescent apoptotic cells for I hour on ice (**A**) or 37 °C (**B**) and binding/ phagocytosis measured by FACS. Data are presented as mean percentage phagocytic DCs +/- sd for triplicate samples The same results were seen in cultures from at least three knockout and control mice.

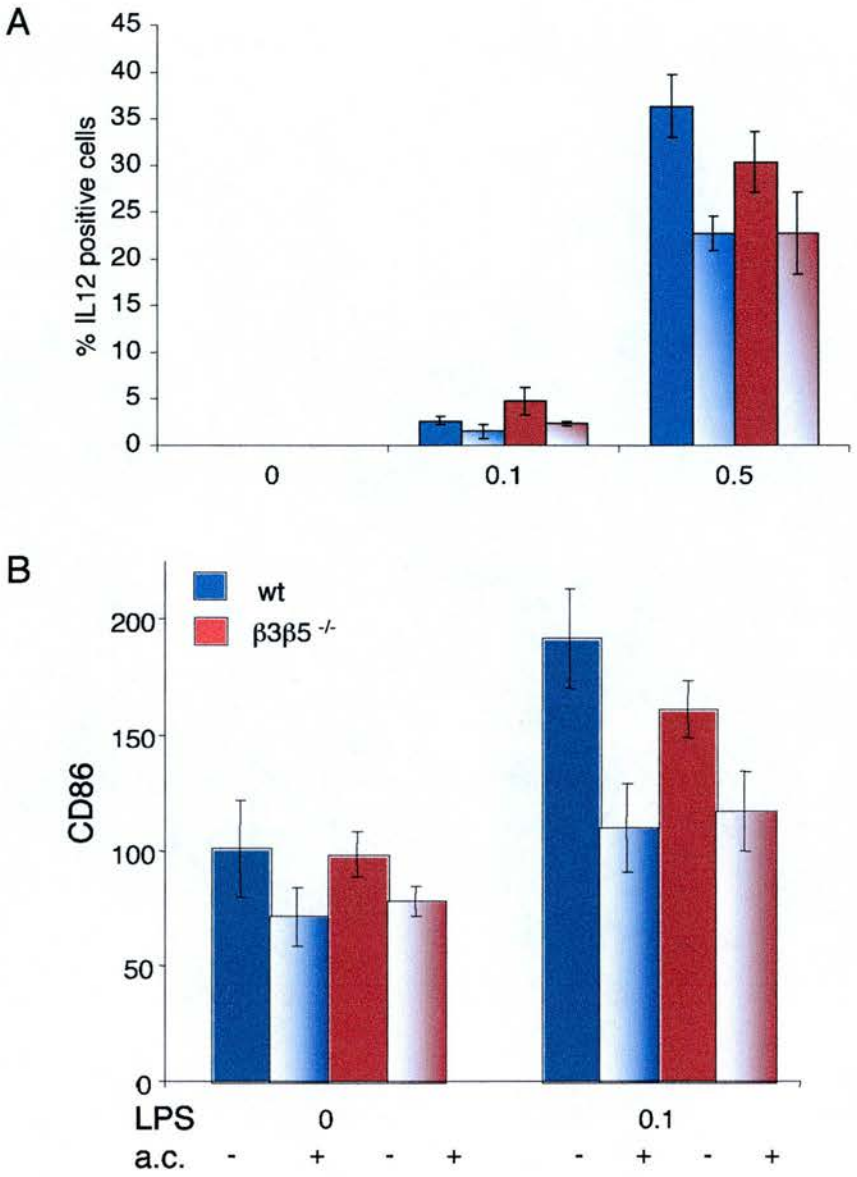


Figure 6.11: Role of β3 and β5 integrins in response of DCs to apoptotic cells. Mouse bone marrow derived DCs from wild type or integrin $\beta3^{-/-}$ $\beta5^{-/-}$ mice were incubated with apoptotic cells (shaded bars) and stimulated with LPS. Maturation was measured by intracellular IL12 or surface CD86 staining. Data are presented as mean percentage positive DCs or mean CD86 GMFI +/- sd for triplicate samples The same results were seen in cultures from at least three knockout and control mice.

6.3.2 Discussion

In this section I have considered the role of two candidate phagocytic recptors invovlved in ac:DC interactions, CD36 and β3/5 integrins (Rubartelli et al. 1997). Experiments using *CD36-/-* DCs confirm previous reports that DCs utilise this molecule to bind apoptotic cells. Interestingly in this murine system binding required a serum component suggesting that murine CD36 does not bind directly to apoptotic cells but via a bridging molecule (likely to be thrombospondin). In contrast, human CD36 has an additional binding site mapped by antibody blockade studies, and shared with malaria-infected erythrocytes, that binds apoptotic cells directly. It is possible that this latter site in murine CD36 binds neither malaria infected erythrocytes or apoptotic cells with high affinity explaining discrepancies found in the role of CD36 in human and mouse studies of both malaria and apoptotic cell clearance. The observation that DCs deficient in CD36 phagocytose normally would suggest redundancy in the scavenger receptor family or compensatory upregulation of alternative receptors. Interestingly, the resistance of *CD36-/-*DCs to OxLDL mediated apoptosis indicates that this particular response shows no redundancy and cannot be compensated for by other receptors.

Our data examining binding to matrix components support published data demonstrating expression of VnR in immature DCs (Albert et al. 1998a). In addition, it suggests that DCs do not express or utilise β1 integrins (as they are relatively poor at binding to laminin) and that fibronectin binding is likely to use αv integrin family members. The differences in fibronectin binding were subtle but may represent either VnR dependent binding or differences in other integrin functions (such αv) modified by the absence of β5/3 signalling. The existence of such integrin 'cross-talk' (Blystone et al. 1999; Boettiger et al. 2001) and data from transfection experiments, demonstrating that αv expression is stabilised by β5/3 (Albert et al. 2001), suggests that defects in the *β5/3-/-* DCs may be due in part to dysfunction in other integrins.

The observation that DC internalisation of apoptotic cells was not blocked by RGDS peptides is in agreement with Rubartellis original study where only Vn-RGD, the natural peptide ligand for VnR, was able to block DC phagocytosis whilst both RGDS and Vn-RGD blocked macrophage internalisation (Rubartelli et al. 1997). The conclusion drawn from these original experiments was that macrophages utilised the TSP binding site of αvβ3 (blocked by RGDS) whilst DCs did not and, in support of this, resting DCs do not to express TSP. An alternative explanation is that DCs utilise an alternative VnR to macrophages, such as αvβ5 as suggested by Albert et al, and that this is blocked by only by Vn-RGD. Establishing the relative use of αvβ3 and αvβ5 in macrophage and DC adhesion will be interesting area of future study and work is currently underway examining single β3 and β5 deficient phagocytes and using Vn-RGD in this murine system and will be particularly important in light of new evidence that DCs express lactadherin (Thery et al. 1999; Thery et al. 2002) shown to bridge αvβ3 to the apoptotic cell in a macrophage system (Hanayama et al. 2002).

However, our data showing no role for β3/5 in binding and phagocytosis in the knockout DCs is rather at odds with evidence derived from antibody blockade experiments. Various possibilities exist explaining this 1) β3/5 integrins are simply not involved in apoptotic cell recognition 2) that under the conditions of our binding assay (ie 4°C) defects are not apparent, particularly likely as integrin binding has been shown to require temperatures of greater than 18°C (Finnemann et al. 1997), 3) that redundancy also exists - ie that αv is able to partner other β integrins, such as β6, to perform these phagocytic tasks or 4) that integrin antibody ligation mediates its effects not by directly blocking the integrin binding site but by signalling to other receptors (including other integrins) and modulate the efficiency with which they bind to apoptotic cells. Interestingly, similar contradictory results have been shown in the role of β3/5 integrins and angiogenesis; antibody blockade experiments directed at β integrins were able to inhibit angiogenesis in murine tumour models whilst

studies using these same *β3/5-/-* mice have shown, if anything, an increase in new vessel formation (Reynolds et al. 2002). Although there is no definite explanation for this apparent paradox, the ability of soluble integrin antibodies to 'transdominantly inhibit' functions of other integrins has been postulated and may also explain this discrepancy. Further studies will be required to determine these possibilities. However, it does appear that apoptotic cell clearance is not limited by recognition by these molecules and is more likely determined by rates of actin polymerisation and cytoskeletal rearrangement, controlled as part of the DC maturation programme.

During the course of this work two papers have been published exploring the role of CD36 and β3/5 integrins in cross-presentation by splenic CD8α+DC (Belz et al. 2002b; Schulz et al. 2002a). Using the same *CD36-/-* and *β3/5-/-* mice these investigators demonstrate that these receptors are not required for internalisation or cross-presentation of osmotically loaded cell associated antigen, suggesting that neither of these receptors are required for phagocytosis or processing of dying cells. These observations are in keeping with our data demonstrating that neither of these receptors are required for the inhibitory response in bone marrow derived DCs. However, CD36 has been identified exclusively on the CD8α+DC subset (Belz et al. 2002b; Iyoda et al. 2002; Schulz et al. 2002a) which preferentially phagocytose apoptotic cells, thus raising interesting questions concerning the role of this receptor in this DC subset.

6.4 Section 3: Role of G-protein coupled signalling molecules.

G protein coupled signalling pathways (Braun et al. 2001) modulate DC cytokine secretion; treatment with pertussis toxin, inhibiting Gαi (and so block Gαi inhibition of adenylate cyclase activity), increases cAMP levels and paradoxically causes spontaneous release of IL12 (He et al. 2000) whilst cholera toxin (activating Gαs directly) inhibits concurrent LPS stimulation (Braun et al. 1999). Apoptotic cell internalisation is modulated by cAMP (Rossi et al. 1998) suggesting a link between apoptotic cell clearance and such a G protein coupled pathway. We have attempted to explore possible roles of such signalling by studying cholera toxin and pertussis toxin effects on apoptotic cell inhibition of DCs.

6.4.1 Results

To confirm a role of G protein receptors in control of cytokine production by LPS treated DCs, DCs were treated with toxins known to modulate G proteins. Our results were consistent with published data; pertussis toxin caused spontaneous release of IL12 and slightly (although not significantly) increased release in response to LPS (Fig 6.12) and cholera toxin inhibited LPS dependent release of IL12 production (Fig 6.12). Treatment of DCs with these inhibitors prior to apoptotic cell/LPS challenge did not affect the ability of apoptotic cells to inhibit DCs (Fig 6.12). These results mitigate against a role for cholera or pertussis sensitive signalling pathways in these events.

6.4.2 Discussion

G protein coupled signalling pathways (Braun et al. 2001) modulate DC cytokine secretion and I confirm that treatment with pertussis toxin causes spontaneous release of IL12 (He et al. 2000) whilst cholera toxin inhibits

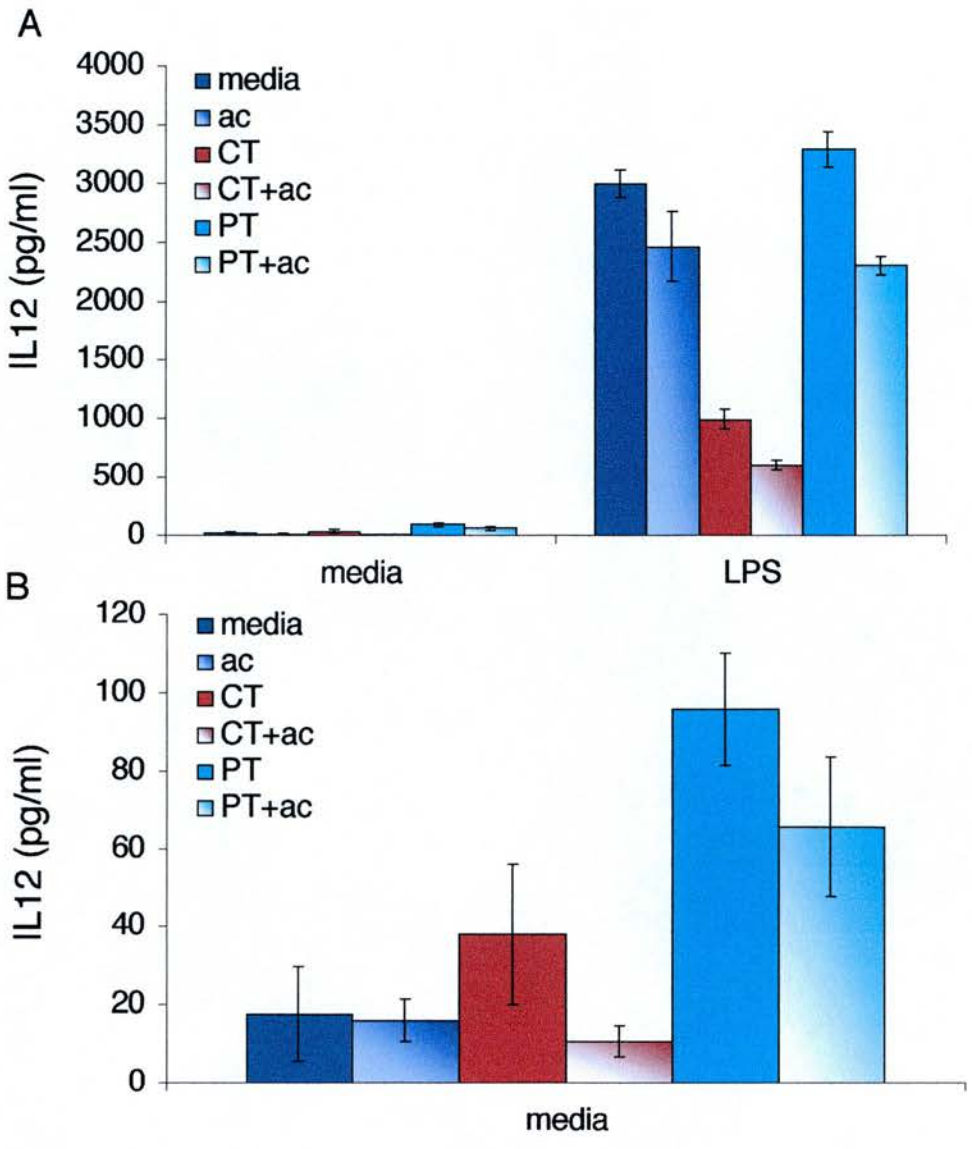


Figure 6.12: Role of G protein signalling in antiinflammatory effects of apoptotic cells. DCs were incubated with apoptotic cells and stimulated with LPS, in the presence or absence of pertussis toxin (0.5μg/ml) or cholera toxin (0.5μg/ml). DC Maturation was measured by production of IL12 in culture supernatants. DCs cocultured with apoptotic cells (shaded bars) produce less IL12 than control DCs (solid blue bar). CT (red bar) inhibits IL12 production in response to LPS and does not effect apoptotic cell inhibition of DCs (red shaded bar). PT causes a small increase in IL12 production in resting DCs and slightly augments LPs responsiveness (turquoise bars) but does not reverse the ability of apoptotic cells to inhibit DCs.

concurrent LPS stimulation (Braun et al. 1999). These observations explain, in part, the ability of cholera to act as a mucosal adjuvant, allowing DC activation but driving immune deviation (Gagliardi et al. 2000) and the ability of Pertussis Toxin to drive Th1 responses and promote autoimmunity in animal models. Resting DCs are thought to be under continuous tonic inhibition mediated by a pertusis sensitive signalling pathway, likely to use cAMP as a second messanger. cAMP has been shown recently to have potent regulatory effects on DC maturation and activation. Interestingly, vitronectin receptors are thought to, when associated with CD47 (integrin assocoiated protein), form an 'ad hoc' seven transmembrane receptor likely to signal by G proteins (Green et al. 1999; Demeure et al. 2000). Thus, the possibility that the vitronectin receptors were acting in concert with CD47 during apoptotic cell phagocytosis to form such 'ad hoc' seven transmembrane receptors mediating the inhibitory response was of interest. However the ability of apoptotic cells to still inhibit both cholera and pertussis treated DCs mitigates against a link between response to apoptotic cells and G protein coupled pathway. However this analysis has not been comprehensive and I believe this area warrants a more detailed analysis of intracellular cAMP signalling in macrophages and DCs after phagocytosis of apoptotic cells.

An additional possibility is that the inhibitory signalling is mediated by CD47 (integrin associated protein) independent of phagocytosis and phagocytic receptors. There is considerable evidence in the literature that this may indeed be the case. TSP, a ligand for CD47, and CD47 antibody ligation have been shown to result in similar patterns of cytokine modulation in DCs, preferentially inhibiting IL12 production (Armant et al. 1999). Also, the malaria infected erythrocytes used to define the role of CD36 by Urban et al (Urban et al. 1999), also bind TSP and may possibly also ligate CD47 on DCs. It is possible that both malaria infected erythrocytes and apoptotic cells complex TSP on their surface and that TSP has numerous important functions. Firstly, it bridges the phagocytic receptors CD36 and/or $\alpha v\beta 3/5$ to an unidentified ligand on the apoptotic cell; secondly, it initiates integrin cross-talk upregulating integrin

function and their recruitment to the 'phagocytic synapse' and finally, by ligating CD47 initiates the inhibitory effects of both apoptotic cells and malaria infected erythrocytes.

6.5 Summary

In summary, since in both *CD36-/-* and *β5/3-/-* DCs apoptotic cells are able to inhibit maturation, it suggests an alternative mechanism independent of these receptors. The fact that phagocytosis can still proceed normally implies that alternative receptors are used and that these too may be capable of signalling the inhibitory phenomenon. Alternatively, the inhibitory phenomenon may be linked to common signalling molecules recruited independently to the phagocytic synapse (such as CD47) or originate distal to receptor ligation from processes (such as p130cas/CRKII/DOCK180/Rac/Cdc42) involved in actin-cytoskeleton rearrangement required for phagocytosis. Determining the mechanism by which apoptotic cells inhibit both macrophages and DCs will be important work in the future.

CHAPTER 7: MACROPHAGES REPROGRAMMED WITH APOPTOTIC CELLS MODULATE BYSTANDER DENDRITIC CELLS

7.1 Introduction

The migratory capacity of DCs is vital for their function. Importantly, DCs are able to 'interpret' the microenvironment in which they first encounter antigen and, by the phenotype they adopt on migration to lymphoid tissue, communicate information concerning the state of the periphery to T cells. Thus, despite a spatial separation of naïve T cells from peripheral tissue, DCs constantly deliver contextual information that helps initiate an appropriate adaptive immune response. Hence, identification of tissue cues able to modulate DCs is of increasing interest. These include endogenous 'danger signals' such as hyaluronan, a matrix component found in inflammatory tissue (Termeer et al. 2002) and necrotic cells (Gallucci et al. 1999; Sauter et al. 2000). In contrast, recently identified tissue factors may be able to deliver 'safety' signals; TSP (Demeure et al. 2000) (which binds CD47), CRP (Gershov et al. 2000) (and other pentraxins) and tissue derived cytokines such as TGF-β (Letterio et al. 1998; Gershov et al. 2000) and IL10 (Fiorentino et al. 1991), can all modulate DC activation, inhibiting IL12 production and initiation of immune responses. Of particular interest is the identification of leukocyte derived factors which modulate bystander DCs; monocyte (Reddy et al. 1997) and T cell (Kato et al. 2001) derived conditioned media contain a variety of components including CD40L, IL1, IL6 and TNFα which mature DCs and factors derived from infected keratinocytes are able to stimulate bystander uninfected Langerhans cells. Thus we should not consider DC functions, including clearance of apoptotic cells, in isolation but rather alongside other leucocytes or in the context of tissue in which it occurs.

Previous chapters have focused on DC interactions with apoptotic cells and demonstrate that only DCs that phagocytose are inhibited with bystander cells remaining unaffected. However, it is becoming apparent that there may be systemic immunosuppressive effects of apoptotic cells (Yoshida et al. 1998).

Therefore it seems likely that cells other than DCs initiate this systemic imunosuppression, generating signals affecting all immune cells. Macrophages share the tissue micro-environment with DCs and because of the high efficiency with which they phagocytose apoptotic cells, their relative numbers and their capacity to secrete factors able to act systemically, they are likely to make a significant contribution to the outcome of apoptotic cell clearance *in vivo*. Interestingly, the presence of contaminating macrophages in *in vitro* cultures appears to retard DC maturation (via secretion of factors including TGF-β) (Yamaguchi et al. 1997) and also inhibits cross-presentation, implicating macrophages in indirectly downregulating adaptive responses driven by DCs (Albert et al. 1998b).

This chapter will investigate the interactions of DCs with macrophages in vitro, with particular emphasis on the effects that macrophages internalising apoptotic cells have on bystander DC maturation.

7.2 Results

7.2.1 Immature DCs can compete with macrophages to phagocytose apoptotic cells *in vitro*

In previous studies, failure of cross-presentation of apoptotic cells in the presence of macrophages has been attributed, at least in part, to antigen sequestration. However, it has been observed that immature DCs are highly phagocytic with up to 50% of immature murine DCs able to internalise apoptotic cells in vitro. Thus, to test the ability of macrophages to prevent DCs from internalising apoptotic cells we established a co-culture system. Green fluorescent-labelled immature DCs were transferred onto a monolayer of murine bone marrow macrophages at ratio of 1:1. To these cultures were added red fluorescent apoptotic human neutrophils and phagocytosis observed after 1h by fluorescent microscopy (Fig 7.1). When DCs were cultured with

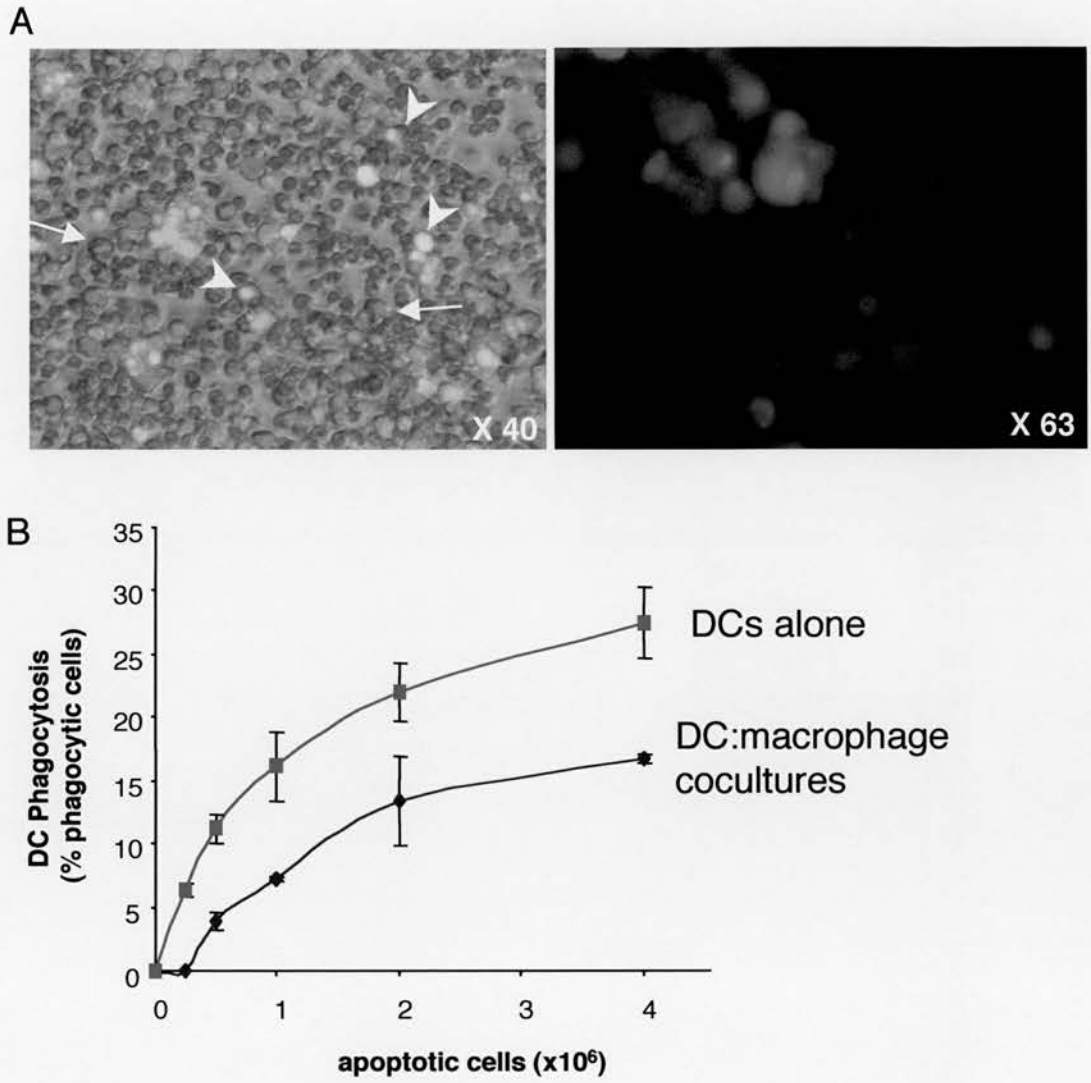


Figure 7.1: Phagocytosis in DC: macrophage co cultures.
Fluorescent green mouse bone marrow derived DCs (green) were cultured with unlabelled macrophages and increasing numbers of red fluorescent apoptotic cells. **A:** DCs can be seen to internalise red apoptotic cells (arrowheads) despite a monolayer of macrophages. Note apoptotic cells can also be seen inside macrophages (arrows). **B:** Phagocytosis of apoptotic cells by DCs was quantified by FACS analysis. Figures show mean percentage phagocytic DCs +/- s.d. for triplicate measurements and representative of three similar experments.

macrophages at a ratio of 1 DC: 2 macrophages inhibition of phagocytosis remained constant at approximately 50% despite varying the phagocyte: apoptotic cell ratio from 3:1 to 1:3 (Fig 7.1). Hence macrophages and immature DCs appear to compete for antigen and the fate of the antigen will depend on the relative numbers of apoptotic cells, macrophages and DCs. Curiously, even after 24 hours of culture, DCs could still be seen to be internalising apoptotic cells, a process that had ceased when DCs were cultured without macrophages (data not shown). Thus macrophages did not completely sequester apoptotic cells away from DCs but rather appeared to prolong their phagocytic lifespan, militating against sequestration of antigen being the sole contributing factor to failure of cross presentation *in vitro* when they contaminate cultures.

7.2.2 Macrophages inhibit T cell proliferation which is in part due to a net inhibitory effect on dendritic cells

Macrophages added to a DC: T cell co-culture inhibited T cell proliferation as assessed by thymidine incorporation (Fig 7.2) confirming previous reports that macrophages can inhibit T cell proliferation (Mills 1991). One model suggests that macrophages act directly on T cells to suppress T cell proliferation. However, I hypothesised that macrophages might suppress DC maturation. To investigate this I chose to culture DCs either alone or with macrophages and assessed DC activation induced by LPS by studying CD86 expression or IL12 production.

Upon treatment with LPS DCs mature and produce large amounts of IL12. Macrophages have been shown to modulate DC activation especially when cultured with immature proliferating bone marrow DCs (Yamaguchi et al. 1997). In confirmation of this co-culturing day 6 DCs with macrophages decreased the release of IL12 in response to LPS (Fig 7.3) suggesting that macrophages could inhibit DCs. Interestingly, this inhibition appeared to be mediated by a contact dependent mechanism, and was not seen when DCs and macrophages were

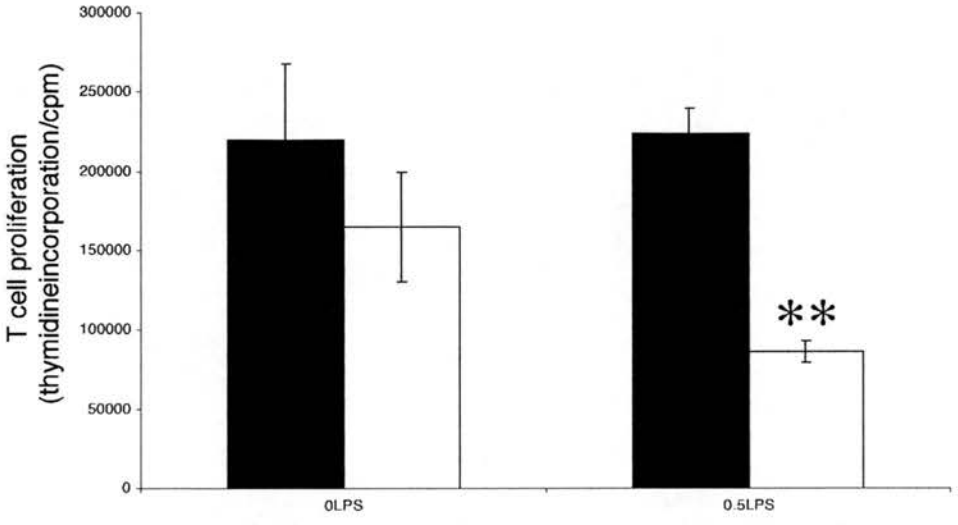


Figure 7.2: Macrophages inhibit T cell proliferation. DCs were, pulsed with OVA peptide and cultured with CD4+ T cells from OVA TCR transgenic (DO11.10)mice in the absence of macrophages (black bars), or in the presence of macrophages (white bars) +/- stimulation by LPS (O.5μg/ml). Macrophages and DCs were separated by a transwell membrane. T cell proliferation was measured by thymidine incorporation (+/-s.d of triplicate cultures).

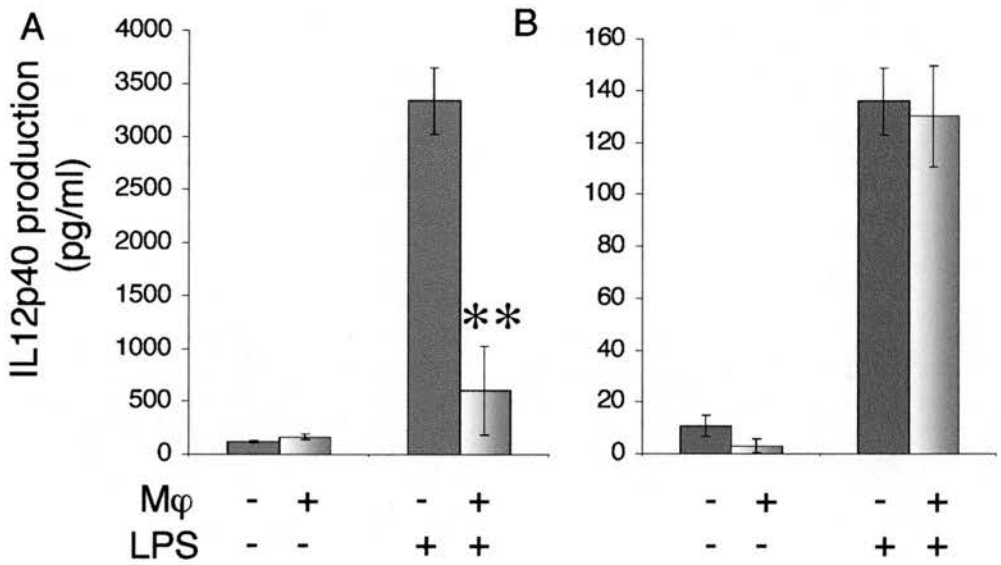


Figure 7.3: Macrophage inhibition of DCs requires cell surface contact. DCs were cultured with macrophages directly (**A**) or separated by a transwell (**B**), matured with LPS and levels of IL12 in culture supernatant measured by ELISA. Figures show mean cytokine levels +/- s.d. for triplicate measurements

separated across a semi-permeable transwell membrane (Fig 7.3) suggesting a role for a membrane receptor or a membrane bound cytokine such as TGFβ, known to be trapped and activated on the surface of macrophages (Murphy-Ullrich et al. 1992).

7.2.3 Apoptotic cell primed macrophages can inhibit dendritic cells in a contact independent manner

Macrophages internalising apoptotic cells have been shown to downregulate proinflammatory cytokine production and upregulate production of important anti-inflammatory mediators including TGF-β (Fadok et al. 1998a; Fadok et al. 1998b). We therefore hypothesised that internalisation of apoptotic cells by macrophages might alter their ability to modulate bystander DCs either by decreasing production of proinflammatory cytokines or causing release of anti-inflammatory mediators. To test the ability of apoptotic cells to modulate macrophages and subsequent DC activation we first allowed macrophages to interact with apoptotic cells for 2 hours, washed off non internalised dead cells and allowed the macrophages to condition their medium for 24 hours. To this conditioned media (which we termed acMo) DCs were added separated from macrophages by a media permeable transwell membrane. DCs, when bathed in this acMo, were inhibited in a contact independent manner, an effect not seen in the control macrophage conditioned media (Fig 7.4). In addition this acMo was less effective than MoCM at inducing CD86 expression on DCs in supernatant transfer experiments (Fig 7.5). This suggested that the acMo either contained less proinflammatory cytokine or produced increased amounts of additional anti-inflammatory mediators. However, neutralisation of TGF-β in acMo in LPS stimulated cultures did not restore the ability of acMo to stimulate CD86 expression suggesting that this was not the active agent (Fig 7.5). This observation suggests that acMo was less stimulatory than MoCM because the pre-treatment of macrophages with apoptotic cells inhibits secretion of proinflammatory factors.

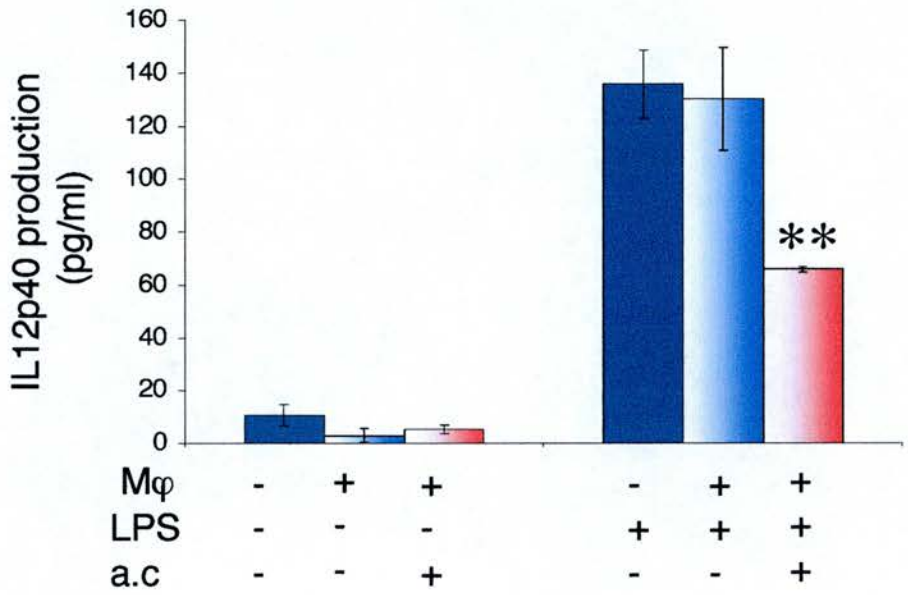


Figure 7.4: Conditioned medium from macrophages treated with apoptotic cells inhibits IL12 production by DCs. Mouse bone marrow derived macrophages were unstimulated or incubated with apoptotic cells, with or without LPS (0.1 μg/ml) and culture supernatant harvested after 24 hours. These conditioned supernatants were added to immature DC cultures. IL12 production by DCs was measured by ELISA after a further 24 hours culture. Figures show mean cytokine levels +/- s.d. for triplicate measurements

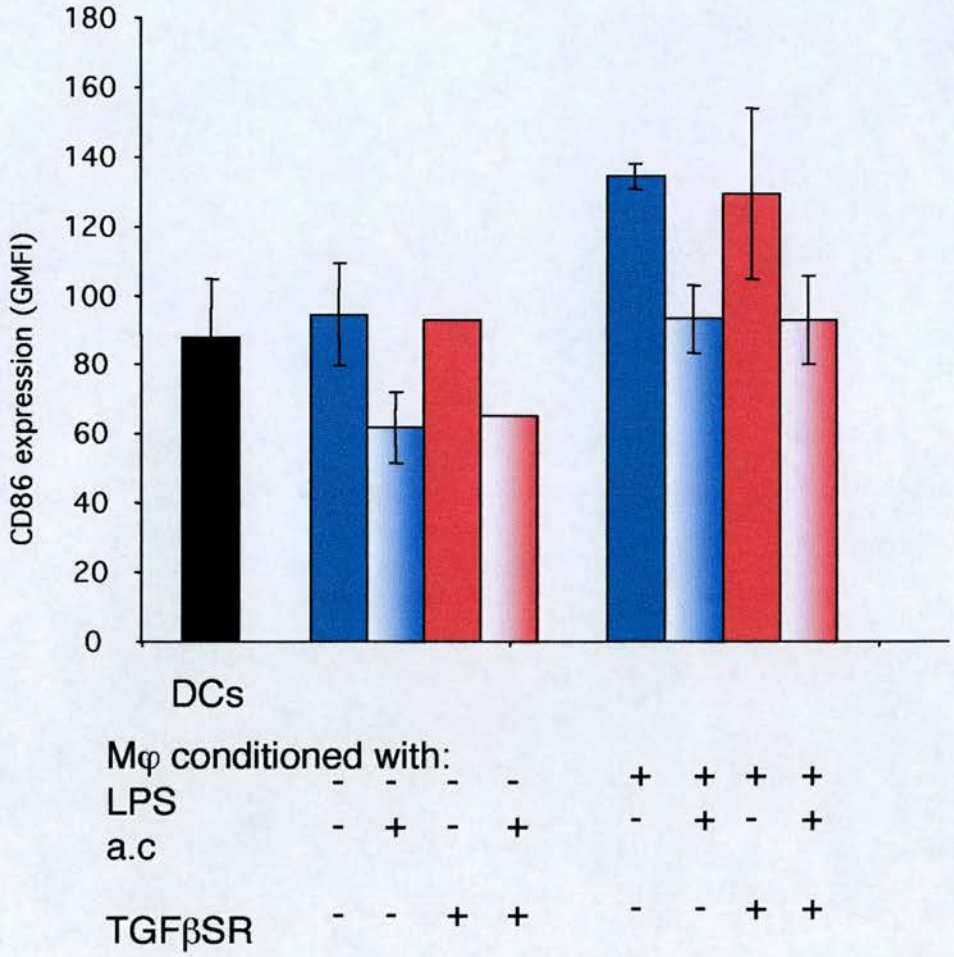


Figure 7.5: Conditioned medium from macrophages treated with apoptotic cells inhibits CD86 expression on DCs. Mouse bone marrow derived macrophages were unstimulated or incubated with apoptotic cells and / or LPS (0.1 μg/ml) and culture supernatant harvested after 24 hours. These conditioned supernatants were added to immature DC cultures, with or without TGF-β soluble receptor (TGFβSR). CD86 surface expression on DCs was measured by FACS after a further 24 hours culture. Figures show mean GMFI +/- s.d. for triplicate measurements. acMo appears to inhibit CD86 expression on resting DCs. Furthermore MoCM but not acMo from LPS stimulated macrophages can induce weak upregulation of CD86 expression. Neutralisation of TGFβ does not reverse this difference.

To further assess if macrophages ability to directly stimulate DCs was modulated by phagocytosis we chose to study the effects of secreted factors from macrophages stimulated with LPS. To prevent direct effects of LPS DCs, DCs from C3H/HeJ mice were used. These mice have a functional mutation in TLR4 rendering them resistant to LPS (Hoshino et al. 1999; Qureshi et al. 1999). Stimulation of these DCs with media conditioned from LPS stimulated macrophages caused release of small amounts of IL12 (Fig 7.6) and weak upregulation of CD86, demonstrating that macrophage conditioned media did indeed contain factors that could indirectly activate bystander DCs. To increase IL12 production the cultures were also stimulated with IFNγ/LPS. Pre-treatment of macrophages with apoptotic cells in these IFNγ/LPS cultures also reduced IL12 production (Fig 7.6). Taken together these data suggest that failure of production of proinflammatory DC maturing cytokines by macrophages that have internalised apoptotic cells is a key factor and that this along with tonic inhibition by contact dependent mechanism contribute to the inhibitory effect of macrophages that have internalised apoptotic cells.

7.3 Discussion

These data demonstrate that DCs continue to phagocytose apoptotic cells in the presence of macrophages. Furthermore, production of cytokines and stimulation of T cell proliferation by DCs were modulated by macrophage contact. These observations confirm previous reports that macrophages have strong ability to suppress T cell proliferation (Mills 1991) and suggest that some of these suppressor effects are in part due to inhibition of DCs. This suppressor activity was greater if macrophages were pre-treated with apoptotic cells. Also the data presented suggests that macrophages secrete proinflammatory mediators and that it is the failure of macrophages that have internalised apoptotic cells to produce proinflammatory factors that is a key contributor to an anti-inflammatory outcome.

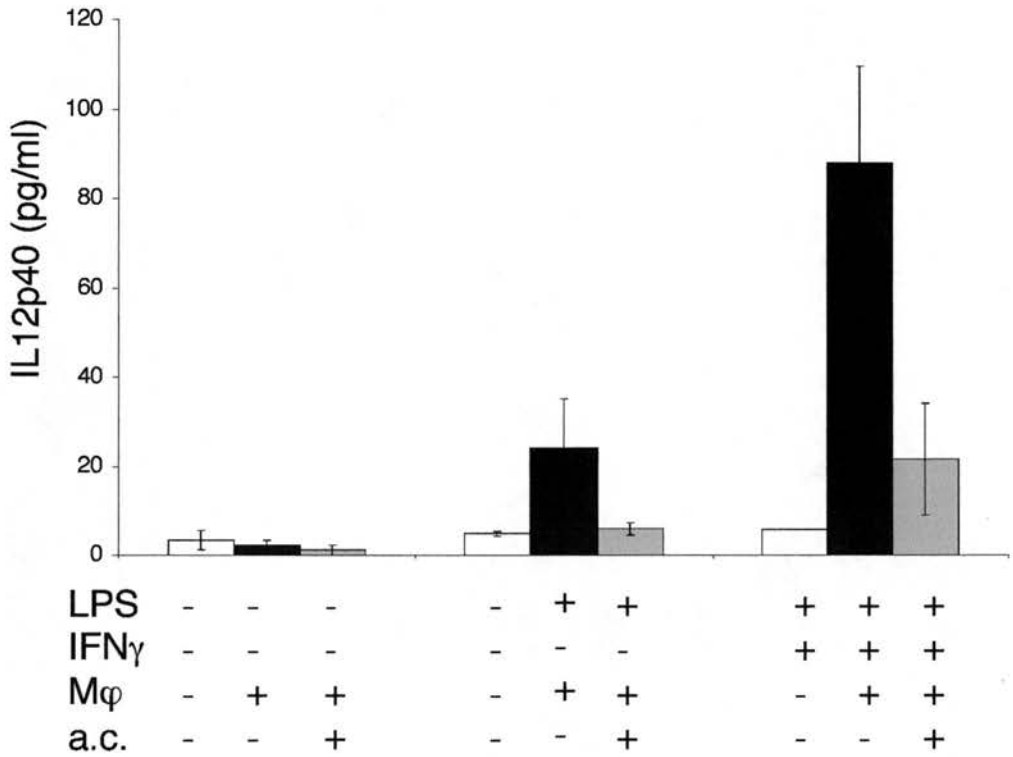


Figure 7.6: Activation of bystander DCs by apoptotic cell treated macrophages. Mouse bone marrow derived macrophages were unstimulated or incubated with apoptotic cells and / or LPS (0.1 μg/ml) and IFN-γ 100U/ml). DCs from C3H/HeJ mice were cultured in transwell inserts over these macrophage cultures for 24 hours, and culture supernatants harvested and IL12 measured by ELISA. Figures show mean cytokine levels +/- s.d. for triplicate measurements. LPS activated macrophages induce low levels of IL12 from bystander LPS unresponsive DCs which can be augmented by IFN-γ priming. Macrophages that have phagocytosed apoptotic cells induce less bystander activation .

Despite high efficiency at internalising apoptotic cells, the ability of DCs to cross-present apoptotic-derived antigen has proved to be a relatively inefficient process hindering their use as source of antigen for immunotherapy. Cross-presentation *in vitro* is inhibited by the presence of contaminating macrophages, thought to 'sequester' apoptotic-derived antigen (Albert et al. 1998b). However, the relative numbers of macrophages, DCs and apoptotic cells will vary in different sites (Hume et al. 1983; Inaba et al. 1994; Hume et al. 2002) and throughout the inflammatory process; resident tissue macrophages and DCs will be present in low numbers in the early stages of inflammation whilst recruited macrophages will arrive in large numbers in the later stages of inflammation when they will heavily outnumber DCs. Thus, the identity of the phagocytes engulfing apoptotic cells *in vivo* may vary as relative numbers of DCs and macrophages change during the highly dynamic process of inflammation and may be difficult to model *in vitro*. However, the data presented from this static assay suggests that macrophages do indeed decrease the number of DCs that internalise apoptotic cells. Furthermore, macrophages internalise large numbers of dying cells efficiently and rapidly and outnumber DCs in many tissues and suggests that, on balance, the majority of clearance of dying cells *in vivo* will be performed by macrophages. Nonetheless, DCs do internalise some apoptotic cells in the presence of macrophages and therefore antigen sequestration alone cannot explain how macrophages prevent crosspresentation.

The origin of activatory and inhibitory cues in vivo depends on the tissue microenvironment, with contributions from resident tissue cells and recruited leucocytes. Macrophages exist in many peripheral tissue with higher frequency than DCs and macrophage derived cytokines and chemokines will make a significant contribution to the tissue microenvironment in which DCs first encounter antigen. It is known that macrophages inhibit bystander T cell proliferation and these data confirm that such inhibition is in part due to suppression of dendritic cell function. Furthermore, macrophages and monocytes interacting with apoptotic cells are reprogrammed and upon further

stimulus by bacterial products switch from producing inflammatory cytokines such as TNFα to production of antinflammatory cytokines such as TGF-β and PGE2 (Fadok et al. 1998a) (Fig 7.7). Interestingly, the anti-inflammatory action of phagocytosing macrophages appears to 'spread' to bystander macrophages *in vitro*, in part through the actions of TGF-β. An attractive possibility is that macrophages recruited to phagocytose dying cells in the inflammatory site are also able to 'spread' their suppressive effects to bystander immature dendritic cells in local tissue and via release of a 'cocktail' of inhibitory cytokines.

However, macrophages may also secrete factors such as TNFα, IL1 and IL6 that augment or at least 'license' DC maturation and a TGF-β vs. TNFα 'switch' has been suggested as being important in bone marrow DC cultures (Yamaguchi et al. 1997). The effects of proinflammatory stimuli (TNFα) on DCs were dominant over the anti-inflammatory mediator (TGF-β) suggesting that the balance of the inhibitory and activatory cytokines produced by macrophages that is a key factor determining the phenotype of bystander DCs (Fig 7.8). Thus the inhibitory effects appear to be accentuated in macrophages that have internalised apoptotic cells because of decreased production of pro-inflammatory TNFα.

Failure of macrophages appropriately to switch off production of proinflammatory cytokines does correlate to susceptibility to autoimmunity; for example mice whose macrophages are deficient in inhibitory Fc receptors are prone to autoimmunity (Takai 2002) and interestingly, macrophages in the Axl-Mer-Tyro deficient mice produce high levels of TNFα and, unlike normal tissue macrophages, may contribute to the autoimmune phenotype by 'licensing' DC activation in tissue (Lu, Q. et al. 2001; Scott et al. 2001). It is interesting to hypothesise that such high levels of TNFα are actually due to perturbations in apoptotic cell driven 'reprogramming' of macrophages and will be of interest in the future. In view of the potent immunosuppressive effects of macrophages, their infiltration into tumours and inflammatory sites, these data would suggest

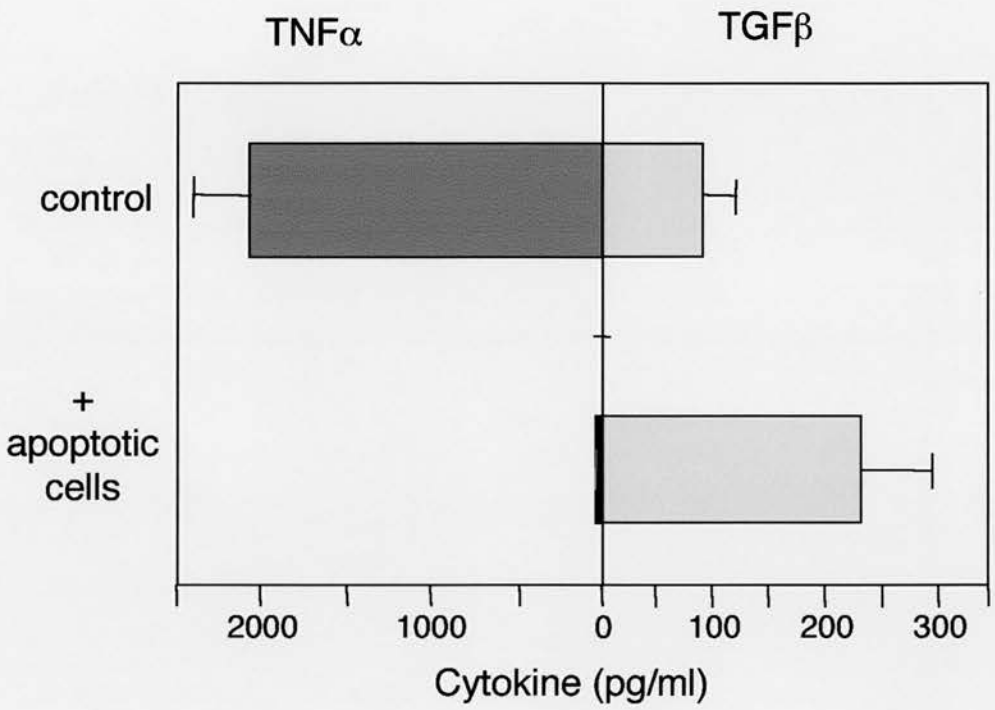


Figure 7.7: Macrophage reprogramming following ingestion of apoptotic cells. Mouse bone marrow derived macrophages were incubated with apoptotic cells for 2 hours or untreated, and then stimulated with LPS (1μg/ml). Supernatants were collected 24 hours after LPS treatment and TNF-α and TGF-β levels determined by ELISA. Data are shown as mean cytokine levels +/- s.d. for triplicate measurements. The same results have been seen in over 5 independent experiments. Macrophages that have internalised apoptotic cells switch off production of TNFα and increase production of TGFβ. (Data courtesy of Mark Lucas)

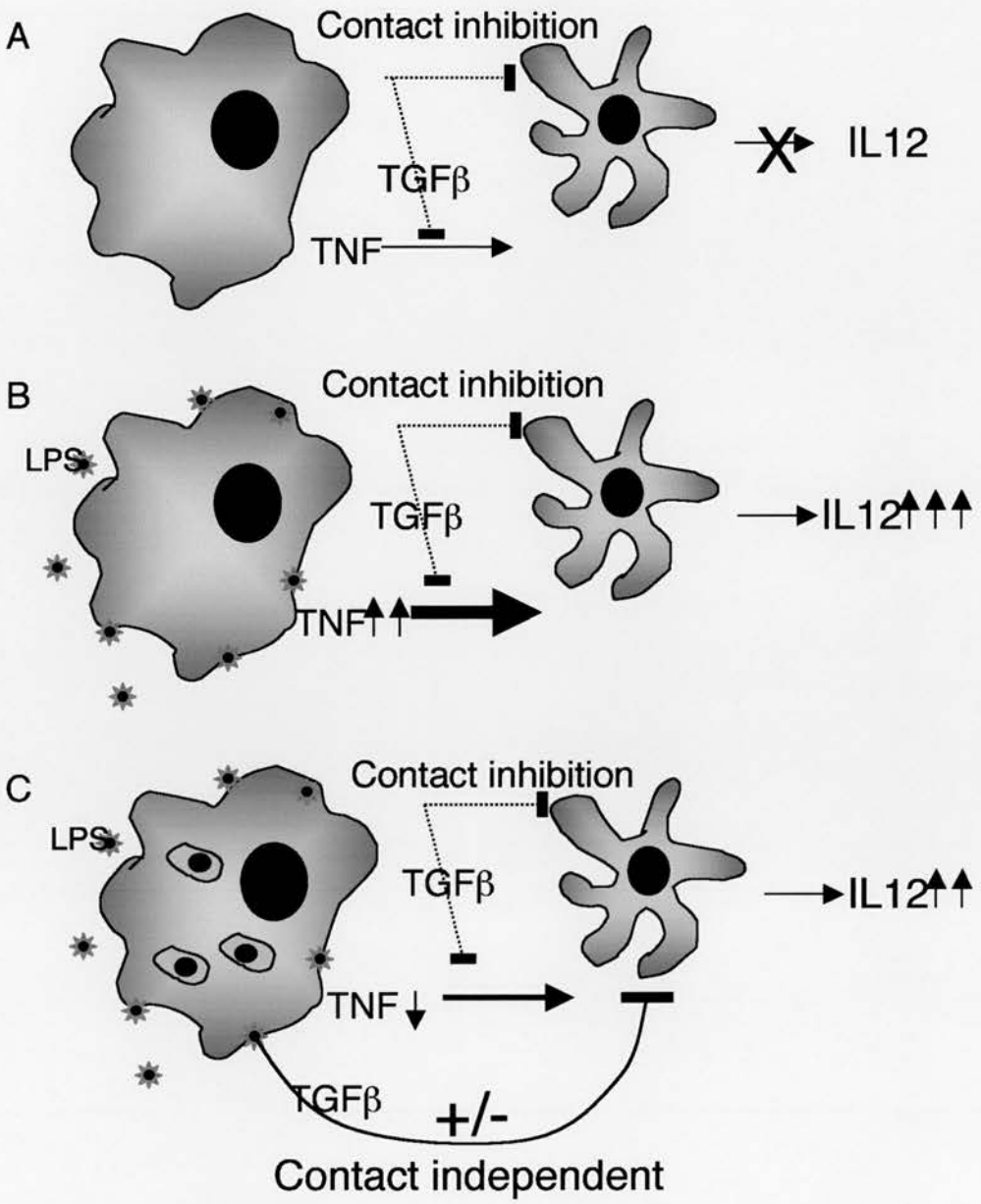


Figure 7.8: Model of macrophage: DC interactions in inflammatory sites

A: Resting macrophages make small amounts of proinflammatory mediators such as TNFα but also inhibit bystander DCs via TGFβ production and a contact dependent mechanism.

B: In a LPS activated environment macrophage production of TNFα licenses DCs to produce large amounts of IL12

C: Macrophages that have internalised apoptotic cells do not produce TNFα and fail to license bystander DCs to produce IL12 which then rely solely on direct activation by LPS. Furthermore, apoptotic cell treated macrophages may upregulate both their contact dependent and contact independent capacity to inhibit DCs.

that they should not be forgotten as an important target for modulation when establishing immunotherapy to apoptotic derived tumour or self-antigen.

CHAPTER 8: DISCUSSION

8.1 Introduction

This thesis has considered the inhibitory consequences of cell death focusing particularly on the dendritic cell. It has explored the role of the DC as an innate immune player, removing dying cells and particulate matter either by endocytosis or phagocytosis. These data presented demonstrate that internalisation of apoptotic cells by DCs modulates their subsequent activation, specifically inhibiting IL12 secretion and upregulation of the costimulatory molecule CD86 and renders them relatively poor antigen presenting cells. In addition, it has explored the possible role of cytokines and phagocytic receptors in signalling these anti-inflammatory responses. Finally, I have attempted to put these DCs back into context – either alongside their phagocytic partners, macrophages, or by studying them *in vivo*.

8.2 Discussion

8.2.1 Apoptotic cells phagocytosis and modulation of DCs

As demonstrated here, apoptotic cells are capable of modulating DCs both directly and via their ability to 'reprogram' macrophages. The direct effects on DCs are closely linked with phagocytosis, implicating ligation of specific phagocytic receptors in propagating the inhibitory signal. However, examination of DCs generated from mice deficient in CD36 and β3/5 integrins, the candidate DC receptors for apoptotic cells implicated in antibody blockade experiments, (Rubartelli et al. 1997; Albert et al. 1998a) demonstrated no defects in either phagocytosis or inhibition by apoptotic cells. It is likely therefore, that DC phagocytosis of dying cells is a more complex and orchestrated event than these original experiments indicated and, similar to the macrophage, further study may identify a variety of additional receptors used by DCs.

Complementarity studies, combining knockouts and antibody blockade, will be required to establish the identity and exact roles of these and other receptors that constitute the DC's 'phagocytic synapse'. Such complementarity strategies may also be required to identify the receptor, at the surface or within the phagolysosome, from which the inhibitory signal originates. Alternatively, inhibition may not be generated from these proximal surface receptor and recognition events but occur distally, emanating from conserved parts of the phagocytic signalling pathway and machinery (eg Rac activation). Indeed, work studying modulation of IL12 secretion by macrophages (Mosser et al. 1999) would support both common and receptor specific inhibitory effects of phagocytosis. A final possibility is that apoptotic cells, acting as 'Trojan horses' for TGF-β (Chen et al. 2001) and other mediators, directly inhibit the phagocytes that internalise them.

8.2.2 DC phagocytosis and implications for cross priming

DCs are scavengers (Austyn 1996), able to phagocytose dying cells. Such cells are likely to be a significant source of antigen provided to DCs *in vivo* and have also been shown to be highly efficient means of delivery of antigen *in vitro*. The realisation that DCs can internalise apoptotic cells and present their derived antigens to the immune system was a fascinating observation (Albert et al. 1998b) especially when considered in the context of class I presentation. It explained a possible route by which uninfected DCs were able to present viral antigens as well as providing a mechanism by which tumour immunity might be initiated. However, this obviously important pathway for priming to such unwanted cellular antigens also provided a possible mechanism by which transplantation antigens might become visible to the recipient immune system and autoimmunity might occur. Hence, although vital for our immune system to function, it appeared likely that DC clearance of apoptotic cells might have a darker side if inappropriately controlled.

These early results encouraged the use of DC loaded with apoptotic cells to crossprime anti-viral (Albert et al. 1998a) and anti-tumour specific T cells (Larsson et al. 2001; Fonteneau et al. 2002). However, it appears that this process is relatively inefficient, requiring a variety of additional factors including strong DC maturation stimuli (such as MCM) and cognate T cell help (to provide CD40 ligation), acting together to induce a specific maturation programme without which tolerance ensues (Albert et al. 2001; Dhodapkar, M. V. et al. 2001). Furthermore, demonstration of cross priming *in vivo* and identification of the responsible cell has proved difficult (den Haan et al. 2000). The difficulties in inducing cross-priming can be explained, in part, by the existence of an inhibitory effect of apoptotic cells on DC maturation as described here. These direct effects of apoptotic cells on DCs imply that they would be relatively poor activators of naïve T cell responses. However,my system is limited in that it does not allow me to determine the amount of antigen that reaches the surface of the DC for presentation to either CD4 or CD8 T cells and this may be an important factor in the final outcome of cross-presentation *in vivo* (Kurts et al. 1998). For example the high efficiency with which apoptotic cells are able to access the endogenous pathway (Inaba et al. 1998) may partly negate their inhibitory effects, especially in situations of 'apoptotic derived antigen overload'. Nevertheless, by studying an externally loaded class II peptide, we have been able to make comparisons between the activation state of ac+ and ac- DCs that would not be possible in conventional class I restricted cross-priming systems, where only ac+DCs would initiate a functional readout. Understanding the mechanism by which DCs are 'modulated' by apoptotic cells should allow us to explore more efficient ways of delivering cellular antigens for cross priming by bypassing these inhibitory signal and favouring maturation (Dhodapkar, K. M. et al. 2002).

8.2.3 ac+DCs in defining 'immunological self' and cross-tolerance

The ability of our immune system to maintain self-tolerance is evidenced by the infrequency with which autoimmunity occurs. Mechanisms maintaining central tolerance are now well understood and beyond the scope of this discussion (Sprent et al. 2002). However, many antigens are not expressed in the developing thymus and additional mechanisms for peripheral tolerance must exist. For such antigens, self-reactive T cells are not deleted centrally and must be deleted or deactivated in the periphery. To this end, it has been realised that regulatory T cells, capable of deactivating effector T cells, are vital (Groux et al. 1997). However, many of these regulatory populations demonstrate some antigen specificity and a memory phenotype implying that they are expanded in an antigen specific manner. It is likely that the initial clonal expansion of such T cells occurs after encounter with DCs. One such candidate is the immature DC, lacking in costimulatory molecule expression and shown *in vivo* to be capable of generating such cells (Dhodapkar, M. V. et al. 2001). Another possibility is that it is a mature but 'modulated' DC that performs this task. In work performed by Albert et al (Albert et al. 2001) they demonstrated that DCs that had phagocytosed apoptotic cells and matured with MCM were in many ways mature, capable of interacting and stimulating naïve T cells. However, the outcome of the interaction was unusual; these 'modulated' DCs, unlike their mature endogenously infected equivalents, did not fully activate influenza specific CTLs unless provided with additional cognate help, and instead tolerized them. The ac+DCs described in this thesis could also be considered as 'modulated' rather than immature as they demonstrate many characteristics of mature/activated DCs, producing TNFα and expressing a variety of surface markers found on mature DCs. However, our ac+DCs are still able to stimulate naïve T cells (albeit to a lesser extent) despite failing to express costimulatory molecule CD86, or produce IL12. These observations would suggest that apoptotic cells modulate DCs, maintain their ability to interact with naïve T cells but driving a distinct 'tolerizing' outcome potentially deleting interacting autoreactive T cells.

In vivo evidence of the existence of such DCs is demonstrated by two descriptions in the rat, (Liu, L. et al. 1998; Trinite et al. 2000). The OX41-/CD4- DCs described in the latter work are relatively poor stimulators of naïve T cells and have been shown to contain inclusions derived from apoptotic intestinal epithelial cells (Huang, F. P. et al. 2000). Such cells were found in the draining lymph and as such were not completely naive as they had presumably received some cue to exit the peripheral tissue. Interestingly, in an elegant *in vitro* model of monocyte/DC trafficking, phagocytosis was able to provide such a stimulus, inducing DCs to reverse transmigrate (analogous to exiting tissue into lymph) (Randolph et al. 1998). It seems likely therefore that the OX41-/CD4- DCs have also been modulated by internalising apoptotic cells ie are mature, migratory but not fully activated. Such migratory subsets probably reach the secondary lymph tissue carrying myriad peripheral tissue antigens. Here they educate T cells, providing a constant source of self-antigen and act to maintain regulatory T cell populations. In this way ac+DCs are a constant reference for the immune system and, as suggested by Steinmann, define 'immunological self' and tolerize to it (Steinman et al. 2000). The data presented would further add to these theories a regulatory control of DC maturation by apoptotic cells, implying that their internalisation is not simply an immunologically null event but actively contributes to maintaining this DC population.

8.2.4 Implications for CD8α+DCs

CD8α+DCs are also thought to have an important role in the maintenance of peripheral tolerance (Belz et al. 2002a). This role of CD8α+DCs was suggested after the original description when these 'lymphoid' DCs were shown have the unusual ability to induce rapid AICD in interacting lympohocytes, raising the possibility that such deletion might remove autoreactive T cells *in vivo* (Suss et al. 1996). Interestingly, apoptosis was crucial in the observed 'killer' phenotype of CD8α+DCs and the conclusion from these observations was that CD8α+DCs actively killed interacting T cells by expressing Fas-L upon their surface.

However, preferential expression of Fas-L by CD8α+DCs has not been supported by subsequent studies using different CD95 antibodies. Instead we would suggest a reinterpretation of these observations and suggest that apoptosis may contribute to this DC phenotype in a more complex way; ac+DCs specifically inhibit IL12 production and costimulatory molecule CD86 expression but continue to make TNFα. Such modulation results in DCs which are relatively poor at stimulating T cell proliferation and prime interacting T cells for rapid AICD upon restimulation. CD8α+DCs, by virtue of their expression of specific receptors for apoptotic cells (CD36), probably clear small numbers of apoptotic cells during normal tissue turnover and larger numbers when rates of apoptosis are high or upon immunisation with dying cells. One such situation is induced during the termination phase of the adaptive immune response when large numbers of redundant effector T cells are deleted by AICD. In this model, internalisation of these dying T cells by the stimulating CD8α+DCs results in down regulation of IL12 production and drives CD8α+DC to adopt a 'killer' phenotype. These CD8α+DCs, perhaps through FasL expression, or other members of the TNF superfamily, that are present or induced by phagocytosis, actively 'kill' interacting T cells, increasing the apoptotic cell load.

Currently, we have little data implicating an absolute requirement for CD36 in this process. However, a precedent for a 'feedforward' role for CD36 function and regulation exists; CD36 is important both in the induction and maintenance of foam cell formation in atheroma (Febbraio et al. 2001) and studies of lipid handling indicate that oxidised lipid, acting as a CD36 ligand, upregulates CD36 expression (Han et al. 1997). We would suggest a similar, but inhibitory, feedforward loop may contribute to the rapid AICD seen in CD8α+DC primed T cell cultures and occurs preferentially with these cells partly because of the efficiency with which they phagocytose dying cells and the inhibitory consequences that ensue. Interestingly, although ligation of CD36 has been shown to be capable of modulating DC function our preliminary data using *CD36-/-*DCs show that it is not an absolute requirement. Furthermore, *CD36-/-*

DCs isolated *ex vivo* demonstrate only a minor defect in apoptotic cell clearance and our preliminary data suggest compensatory mechanisms exist between scavenger receptor family molecules *in vivo*. We favour the concept that it is the ability of DCs to internalise apoptotic cells, rather than the receptor involved that is key. Taken together these data suggest a novel mechanism by which normal clearance of constitutively apoptotic cells might contribute to DC function and the maintenance of the 'killer' CD8α+DC phenotype *in vivo*.

8.2.5 Implications for tumour immunity

Apoptosis is a characteristic of certain tumours and is also the form of cell death induced by many anti-tumour treatments. A major goal for medical science is to understand how we can harness natural immune responses to provoke tumour regression. Induction of tumour specific T cells is one such strategy but is hindered by identification of antigens specific for a particular tumour capable of generating and maintaining tumour reactive T cells. Dying tumour cells are an attractive source of such antigens as most of the antigenic repertoire of a tumour will be represented within them. However, although efficient cross-priming to tumours has been demonstrated in both model animal systems and an increasing number of human trials it is only now apparent that certain 'tricks of the trade', such as vigorous *ex vivo* stimulation of DCs, opsonisation of antigen to increase immunogenicity and careful immunisation regimes, are essential for a successful outcome (Steinman et al. 2001). Furthermore, generation of autoimmunity as a consequence of such immunotherapy strategies is a genuine concern (Ludewig et al. 2000; Gilboa 2001). These data presented partly explain this and also imply that limited apoptosis by a tumour may well be a means of immune evasion. Cell death recruits phagocytes and macrophages are in fact, commonly found infiltrating tumours. Although the role of these tumour macrophages is not fully understood, they are thought to benefit the tumour by providing angiogenic factors. The data presented would suggest they may further benefit the tumour by hindering cross-presentation and possibly

expanding tumour specific Treg cells if dying tumour cells are internalised by DCs. In addition, infiltrating macrophages may also deactivate any bystander DCs carrying appropriate antigens preventing initiation or maintenance of a tumour specific response. Thus, the darker side of ac+DCs and macrophage clearance of dying cells is that it provides a potential means of immunological 'escape' for tumours.

8.2.6 Implications for autoimmunity

Autoimmune diseases are complex and multifactorial, involving, amongst others, abnormalities in B and T cell activation thresholds, lack of regulatory controls and genetic factors (O'Shea et al. 2002; Ohashi 2002; Walker et al. 2002). The models outlined here demonstrate how apoptotic cells might, as a source of self-antigen, prevent rather than initiate autoimmunity. Apoptotic cells as presented to the immune system by DC define 'immunological self' (Steinman et al. 2000), without which autoimmunity would be inevitable. However, autoimmunity does occur, and, in some cases, has been shown to be associated with perturbations in normal apoptotic cell clearance processes (Mevorach 2000; Walport 2000; Rosen et al. 2001). Considering data presented within this thesis, such associations could be explained by a variety of hypotheses (Fig 8.1):

1) *Failure of macrophages to clear apoptotic cells results in excess number of dying cells, which are cleared instead by DCs.* Although this is an attractive initial hypothesis, the observation that the DC that internalises an apoptotic cells is in fact inhibited does not support such a theory. The data presented in this thesis would suggest that autoimmunity would only result if clearance of apoptotic cells became proinflammatory in these circumstances, e.g. as a result of secondary necrosis, DC 'overload' or stimulating tissue derived 'danger signals' able to overcome the inhibitory effects of apoptotic cells.

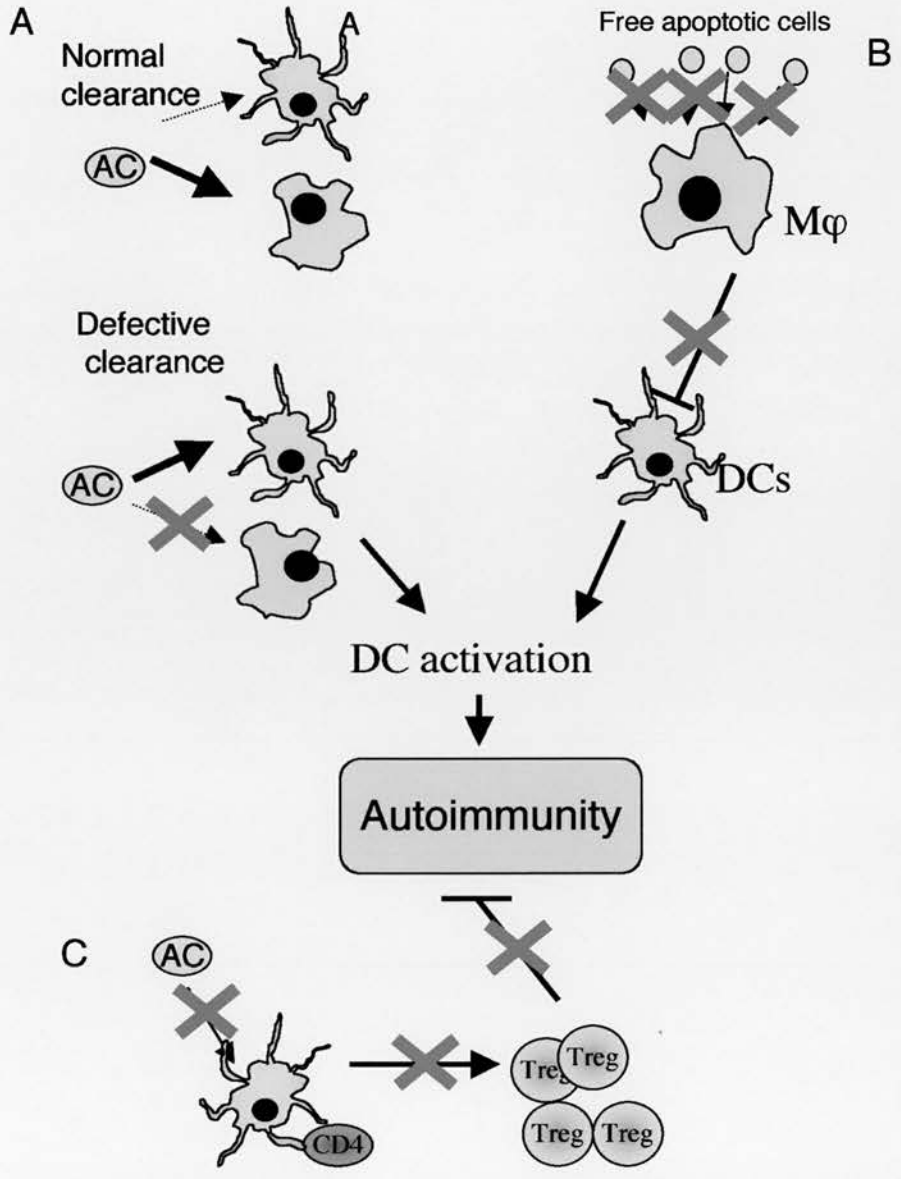


Figure 8.2. Possible mechanisms by which defective apoptotic cell clearance might drive autoimmunity. **A:** Failure of macrophages to clear apoptotic cells results in excess numbers of dying cells, which are cleared by DCs. **B:** Failure of macrophages to clear apoptotic cells results in macrophages that fail to inhibit bystander DCs. **C:** Failure of DC clearance and failed generation of regulatory T cells or deletion of auto-reactive T cells

2) *Failure of macrophages to clear apoptotic cells results in macrophages that fail to inhibit bystander DCs.* As shown, apoptotic cell internalisation 'reprogrammes' macrophages to secrete antinflammatory cytokines able to prevent DC maturation. Failed clearance of apoptotic cells by macrophages, by curtailing their gatekeeper role, might suffice to 'permit' or lower the threshold for bystander DC maturation.

3) *Failure in DC clearance and failure to generate/maintain regulatory T cells or delete autoreactive T cells.* ac+DCs may act primarily to maintain regulatory T cell populations and delete self-reactive T cell clones in the periphery and failure of these regulatory checkpoints because of failed clearance by DC might contribute to autoimmunity.

It is possible that some or all of the mechanisms described contribute to generation or maintenance of self-reactive effector cells found in autoimmune disease.

8.3 Summary: Apoptotic cells, macrophages and modulation of DCs

Together, these data would support a complex model for the role of macrophages, apoptotic cells and DCs in control of immunity (Fig 8.2). Local macrophages, especially in inflammed tissue, would control T cell proliferation by modulating DC function and prevent formation of extra-lymphoid tissue. In addition, macrophages recruited for internalisation of apoptotic cells during the resolution phase of inflammation, would be able to dampen systemic inflammatory responses effectively turning off recruitment of effector lymphocytes, through secretion of factors able to act at distant sites such as TGF-β. In this way macrophages may act as 'gate-keepers' of DC maturation in the periphery and down regulate adaptive immune responses.

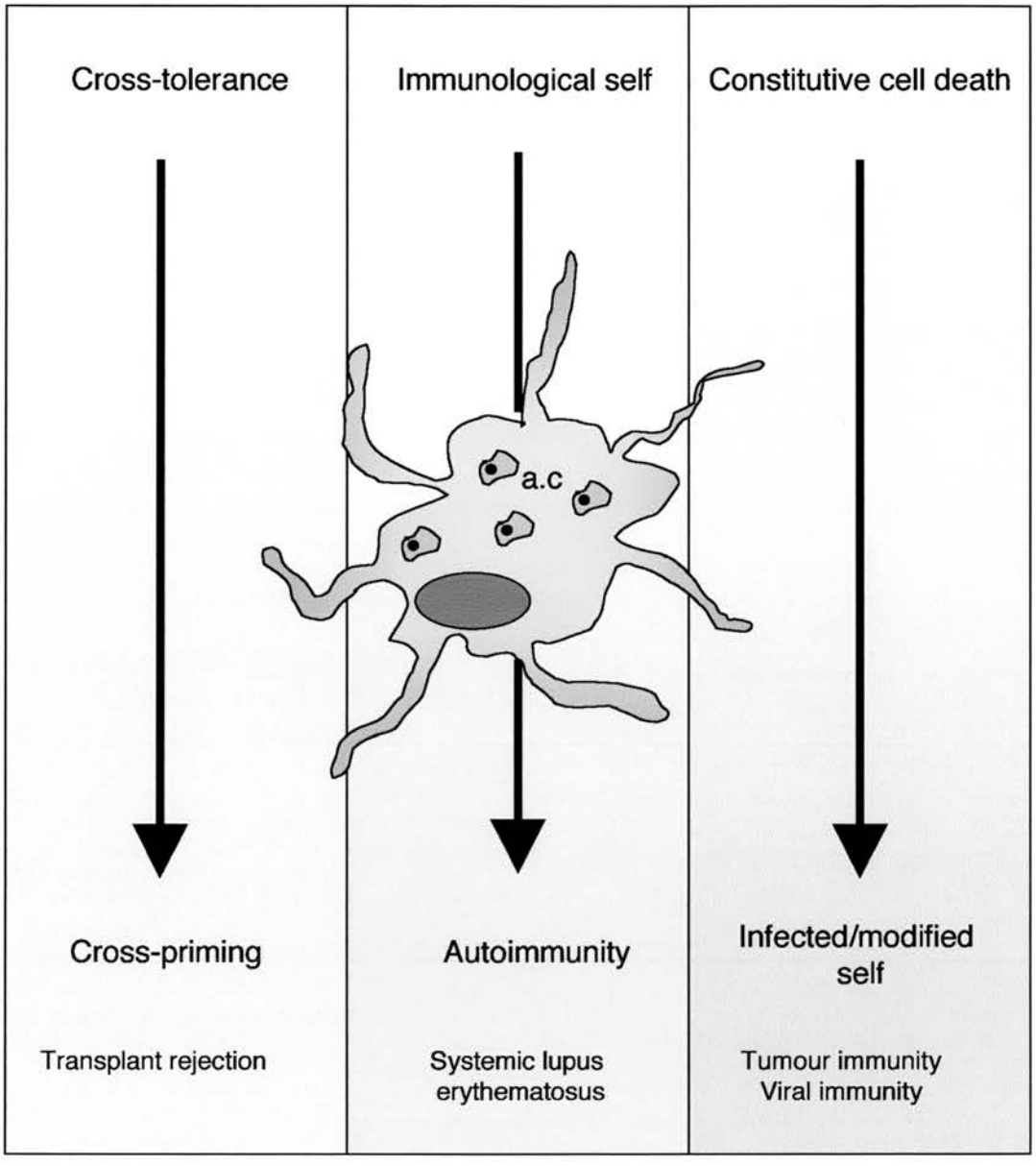


Figure 8.2. Spectrum of outcomes that DCs internalising apoptotic cells might have on the immune system

Also, in normal steady state, DCs loaded with apoptotic antigen would traffic to draining lymph nodes continuously sampling the peripheral tissue. These ac+DCs would fail to mature and hence tolerise/anergise/delete interacting self-reactive T cells. In inflammation, DCs escaping the periphery carrying apoptotic cell derived antigen, because of the direct inhibitory effects of apoptotic cells, would be prohibited from presenting auto-antigens. In contrast, dendritic cells escaping the inflammatory site carrying pathogen-derived antigen, upon encounter of their appropriate cognate T cell, would activate normally. However, this network of control so important in the resolution of inflammation and control of autoimmunity, allows 'escape' of apoptotic tumours from efficient cross-presentation, effectively rendering them invisible to the adaptive immune system.

8.4 Conclusion

"That the organism possess certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organs own elements."
Paul Ehrlich

Dendritic cells are effective scavengers of their local microenvironment, constantly internalising antigen for processing and presentation. Their migratory capacity and unique ability at interacting with naïve T cells to generate both immunity and tolerance is increasingly being understood and has highlighted their importance in bridging the innate and adaptive immune systems.

Phagocytosis of apoptotic cells appears to provide DCs with a constant burden of self-antigen for delivery to the draining lymph nodes. However, the ability of apoptotic cells to actually inhibit the DCs that have internalised them ensures

the safe passage of derived antigen in most cases. In fact these ac+DCs are multifunctional – they constantly provide a source of processed and presented tissue-derived antigens, defining 'immunological self' and are likely to maintain regulatory T cell populations *in vivo*.

However, the darker side of these ac+ DCs must exist. It is likely that they are involved, directly or indirectly, in the induction of autoimmunity and also in transplantation rejection. In addition the ability of certain viruses and other pathogens such as malaria to harness these mechanism for immune evasion is possible. Finally, it is possible to envisage how apoptotic tumour cells might use this portal to 'hijack' DCs, infiltrating the regulatory network and effectively evading the immune system.

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Publications arising from this thesis

Stuart, L., M. Lucas, et al. (2002). "Inhibitory effects of apoptotic cell ingestion upon endotoxin driven myeloid dendritic cell maturation." J Immunol **168**(4): 1627-35.

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Inhibitory Effects of Apoptotic Cell Ingestion upon Endotoxin-Driven Myeloid Dendritic Cell Maturation[1]

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Dendritic cells (DCs) are the sentinels of the immune system, able to interact with both naive and memory T cells. The recent observation that DCs can ingest cells dying by apoptosis has raised the possibility that DCs may, in fact, present self-derived Ags, initiating both autoimmunity and tumor-specific responses, especially if associated with appropriate danger signals. Although the process of ingestion of apoptotic cells has not been shown to induce DC maturation, the exact fate of these phagocytosing DCs remains unclear. In this paper we demonstrate that DCs that ingest apoptotic cells are able to produce TNF-α but have a diminished ability to produce IL-12 in response to external stimuli, a property that corresponds to a failure to up-regulate CD86. By single-cell analysis we demonstrate that these inhibitory effects are restricted to those DCs that have engulfed apoptotic cells, with bystander DCs remaining unaffected. These changes were independent of the production of anti-inflammatory cytokines TGF-β1 and IL-10 and corresponded with a diminished capacity to stimulate naive T cells. Thus, the ingestion of apoptotic cells is not an immunologically null event but is capable of modulating DC maturation. These results have important implications for our understanding of the role of clearance of dying cells by DCs not only in the normal resolution of inflammation but also in control of subsequent immune responses to apoptotic cell-derived Ags. *The Journal of Immunology,* **2002, 168: 1627–1635.**

Dendritic cells (DCs)[3] are the sentinels of the adaptive immune system and have an important role not only in induction of immunity but also in maintenance of tolerance (1). Immature DCs exist in the periphery, where they capture and process exogenous Ag. Upon receipt of maturation stimuli they migrate to draining lymph nodes, a process associated with phenotypic changes, including down-regulation of their Ag-capturing machinery, up-regulation of MHC and costimulatory molecules, and production of IL-12, becoming fully functional APC (2). This maturation process is affected by a variety of endogenous or exogenous factors and can be modeled in vitro by LPS and other bacterial products (3).

Immature DCs acquire Ag by many pathways including uptake of soluble Ag or protein complexes by endocytosis and macropinocytosis and ingestion of entire cells by phagocytosis. Ingestion of certain necrotic cells is capable of inducing DC maturation, while ingesting apoptotic cells fails to activate DCs, appearing to be an immunologically null event (4, 5). However, such DCs are capable of responding to strong external stimuli, such as monocyte-conditioned medium or IFN-γ, to mature and present Ag derived from the ingested apoptotic cells to T cells (6–9). Apoptotic cells are a preferential source of many autoantigens (10), often found localized to apoptotic blebs, and the ability of DCs to present such Ags unchecked might initiate autoimmunity. In support of this, perturbations in apoptotic cell death and clearance of these cells have been shown to contribute to the induction of autoimmunity (11, 12).

However, a growing body of evidence implicates DCs that ingest dying cells in maintaining self-tolerance, by constantly sampling peripheral self Ags and presenting them in a tolerogenic way to the adaptive immune system. Thus, a dichotomy exists in responses of DCs that may be either "friend or foe" (13). The ability of a DC to deliver "signal 2," either as costimulation or IL-12, singly or in combination, appears key in determining subsequent immune responses and is likely to be tightly controlled. Interestingly, the ingestion of apoptotic cells by macrophages generates an active anti-inflammatory response through the production of TGF-β1 and other anti-inflammatory molecules and down-regulates subsequent release of proinflammatory cytokines (14–16). Because of the close lineage relationship of macrophages and myeloid DCs, we postulated that apoptotic cell ingestion by DCs might similarly modulate their effector functions.

In this paper we confirm that immature murine bone marrow-derived DCs ingest apoptotic cells and, after phagocytosis, become functionally distinct. We demonstrate that endotoxin-induced production of IL-12, but not TNF-α, is selectively diminished in DCs that have ingested apoptotic cells. In addition, endotoxin-driven up-regulation of the costimulatory molecule CD86 is inhibited in those DCs that had phagocytosed apoptotic cells, but not in neighboring DCs. We show the functional consequences of these changes by demonstrating that these DCs have a reduced capacity to stimulate T cell proliferation. Thus, phagocytosis of apoptotic cells affects subsequent maturation of DCs in a manner analogous to the anti-inflammatory effects in macrophages, generating mature, $CD86^{low}$ cells that produce less IL-12. These data confirm that the apoptotic cell is not immunologically null, but, by inhibiting DC activation, may contribute to down-regulation of the response to apoptotic cell-derived self Ag and maintenance of self tolerance.

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Received for publication September 6, 2001. Accepted for publication December 12, 2001.

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[1] L.M.S. was supported by Wellcome Trust Clinical Training Fellowship 34842, and J.S. was supported by Wellcome Program Grant 34561.

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[3] Abbreviations used in this paper: DC, dendritic cell; ac, apoptotic cell; SAC, *Staphylococcus aureus* (Cowan strain).

0022-1767/02/$02.00

Materials and Methods

Mice

BALB/c mice were purchased from B & K Universal (Hull, U.K.) and were used at 8 wk for bone marrow-derived DC and macrophage preparation. T cells were isolated from the DO11.10 transgenic mice expressing TCR specific for the chicken OVA peptide, $OVA_{323-339}$, in association with I-A^d. The mice were typed for the presence of the transgene using Abs against CD4 (BD PharMingen, San Diego, CA) and biotin peak 2 (KJ1-26; Scottish Antibody Production Unit, Lanarkshire, U.K.) and streptavidin-PE (BD PharMingen) on PBL.

DC and macrophage culture

Murine cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME, and 15% heat-inactivated FBS unless otherwise stated. DC complete medium also contained 10–15% conditioned supernatant from a hybridoma (gift from Prof. D. Gray, Edinburgh, U.K.) expressing rGM-CSF, resulting in a final GM-CSF concentration of 20–30 ng/ml. This hybridoma also produces IL-10, at a final concentration of ~2 ng/ml, but no TNF-α or IL-12. All culture reagents were obtained from Life Technologies (Grand Island, NY) unless otherwise stated.

DCs were cultured as described previously (17). Briefly, femurs from BALB/c mice were removed, dipped in 70% ethanol for 10 s, and then placed in DC complete medium. Bone marrow was flushed from femurs, and 10 ml of a single-cell suspension of bone marrow cells at 2×10^5/ml was plated in non-tissue culture grade petri dishes. On day 3 an additional 10 ml of fresh medium was added to the cultures. On day 6 half the medium was removed, and the cells were pelleted, resuspended in fresh medium, and added back to the petri dishes. On day 7 nonadherent cells were removed, leaving strongly adherent macrophages on the plate. These cells were pelleted, resuspended at 2×10^5 cells/ml, and replated before use. On day 7 these were a heterogeneous population, 65–80% of the cells having surface phenotype and morphology of immature DCs (Fig. 1*A*), with granulocytes being the main contaminant. Maturation was initiated on day 7 with 0.1–1 μg/ml LPS (*Escherichia coli* serotype 026:B6; Sigma-Aldrich, St. Louis, MO) or 0.02% (w/v) *Staphylococcus aureus* (Cowan strain) (SAC; Calbiochem, La Jolla, CA) (3), and cells were assessed on day 8.

Bone marrow-derived macrophages were cultured in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FBS, and 10% conditioned supernatant from L929 cells, which was changed on day 2 of culture. Macrophages were used for experiments on day 7 of culture.

Generation of apoptotic cells

Neutrophils were extracted from peripheral blood of healthy volunteers as described previously (18). Briefly, blood was separated using dextran sedimentation and a Percoll gradient. This yielded highly pure human neutrophils (>90%), which were allowed to undergo constitutive apoptosis by aging overnight in Iscove's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% autologous serum. After this period the cells were 40–80% apoptotic by cytospin morphology. This method of generating apoptotic bodies was preferred because there was no significant necrosis (<1%) by trypan blue exclusion, as confirmed by annexin-propidium iodide staining and flow cytometry. Apoptotic murine thymocytes were also generated for use in some experiments by treating single-cell suspensions of thymocytes with dexamethasone for 4–6 h. This method yielded apoptotic cells, but these preparations often contained contaminating postapoptotic cells and other nonapoptotic thymic cells. For most experiments cells were stained using a green cell tracker dye (Molecular Probes, Eugene, OR) before overnight culture.

DC-apoptotic cell coculture

Fluorescently labeled apoptotic cells were cocultured with day 7 DCs at a ratio of 2–5:1, apoptotic cells:DC. Interaction of DCs with apoptotic cells was assessed by removing cells after 2 h and staining with allophycocyanin-CD11c for FACS analysis. All FACS analyses were conducted on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Maximum interaction was seen at ratios of 5:1 apoptotic cells/DCs, but the large numbers of uningested apoptotic cells reduced the efficiency of cell sorting, so ratios of 2:1 were used in most experiments. For fluorescent microscopy DCs were grown on chamber well slides, allowed to interact with red fluorescent apoptotic cells, then fixed with 4% paraformaldehyde. Slides were stained in PBS with 0.5% BSA and 0.2% sodium azide with I-A^d-FITC (BD PharMingen) in the presence of 10% normal mouse serum. Slides were mounted and examined under ×63 oil lens using an inverted microscope (Zeiss, New York, NY), and images were captured using Open Lab software (Improvision, Coventry, U.K.) and CoolSnap digital camera (Media Cybernetics, Silver Spring, MD).

T cell proliferation assays

CD4 T cells were isolated from spleens of DO11.10 transgenic mice. In brief, spleens were made into a single-cell suspension by passing through a 53-μm pore size filter in PBS, and debris and red cells were removed by density gradient sedimentation through Lympholyte-M (Cedarlane Laboratories, Ontario, Canada) according to the manufacturer's instructions. Cells were then isolated using L3T4 (CD4) microbeads and the MACS purification system (Miltenyi Biotec, Auburn, CA). Day 7 DCs were cocultured with apoptotic cells for 4 h, stimulated with LPS overnight, and then pulsed with 5 μg/ml OVA peptide ($OVA_{323-339}$; Albachem Laboratories, Edinburgh University, Edinburgh, U.K.) for 2 h. They were then washed thoroughly, and cells were sorted into DCs containing apoptotic cells and those not, using a FACSVantage cell sorter (BD Biosciences). Contaminating apoptotic cells could be excluded from the sort by their smaller size and bright fluorescence. OVA-TCR-transgenic T cells (3×10^5) were cocultured in 24-well tissue culture dishes with varying doses of DCs for 5 days in a final volume of 2 ml. Proliferation was assessed by removing triplicate 100-μl samples pulsed with 1 μCi/well [^{3}H]thymidine (Sigma-Aldrich) for 16 h. Cells were harvested, and thymidine incorporation was measured using a scintillation counter. Interactions were performed in duplicate.

FACS analysis of costimulatory molecules

DCs were cocultured with apoptotic cells for 4 h before overnight stimulation with LPS. Nonadherent cells were then removed from plates and resuspended in PBS with 0.5% BSA and 0.2% sodium azide. Blocking was performed using 10% mouse serum for 15 min, then cells were stained with relevant Abs at 4°C in the dark for 30 min. Cells were then washed and resuspended in 200 μl of FACS wash and analyzed using FACSCalibur and FlowJo software. The following Abs were used (all from BD PharMingen unless otherwise stated): FITC-I-A^d/I-E^d, PE-CD40, PE-CD86, PE-CD54, PE-CD11c, allophycocyanin-CD11c, and FITC-F4/80 (Serotec, Oxford, U.K.). All samples were compared with appropriate isotype controls. The geometric mean fluorescence of cells positive to isotype control was used in analysis unless stated otherwise.

Cytokine detection

For intracellular cytokine staining, cells were cocultured with apoptotic cells for 4 h and stimulated with LPS for 5 h in the presence of GolgiPlug (BD PharMingen) according to the manufacturer's instructions. Cells were harvested and stained for cell surface markers as described above. Cells were then fixed using 4% paraformaldehyde and permeabilized with 2% saponin in PBS with 0.5% BSA, 0.2% sodium azide, and 10% mouse serum while staining with allophycocyanin-TNF-α, IL-10, and IL-12p40/p70 (BD PharMingen). IL-10 could not be reliably detected above background by intracellular staining and so was also measured in the supernatant after 24–48 h of interaction with apoptotic cells and LPS using a Quantakine ELISA kit (R&D Systems, Minneapolis, MN). Interactions were performed in duplicate wells, and triplicate readings of each supernatant were made. Soluble forms of IL-10 and TGF-βR were obtained from R&D Systems and used at the recommended concentrations (1.25 and 0.5 μg/ml, respectively).

Results

Immature murine myeloid DCs ingest apoptotic cells

Day 7 murine myeloid DCs cultured as described above were immature by cell surface phenotype ($CD11c^+$MHC class $II^+CD40^-CD80^{low}CD86^{low}$) and could be matured (as evidenced by up-regulation of costimulatory molecules and MHC class II) over 24 h by the addition of LPS in a dose-dependent manner (Fig. 1*A*). Similar results were seen by stimulation with SAC (data not shown). Apoptotic cells stained with a fluorescent cell-tracker dye were cultured with DCs at a ratio of 5:1, and interactions with DCs were quantified by flow cytometry. In a typical experiment, 2 h after coincubation ~50% of $CD11c^+$ cells had interacted with apoptotic cells. Such interaction was exhibited by <5% of DCs at 4°C, demonstrating that the interaction assay used predominantly detected phagocytosis rather than binding (Fig. 1*B*). Ingestion was further confirmed by fluorescence microscopy (Fig. 1*C*). Similar

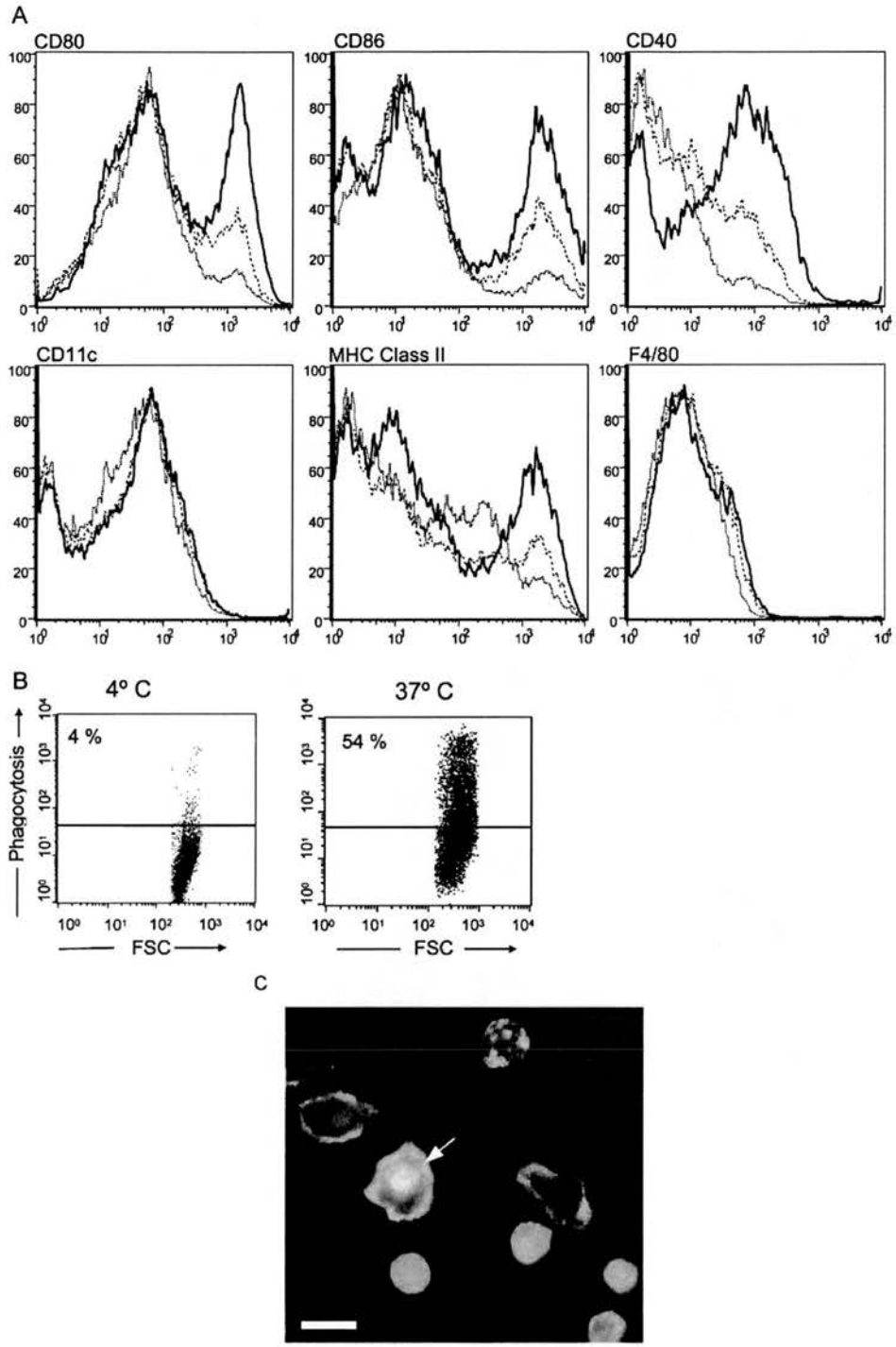

FIGURE 1. Immature murine myeloid DCs ingest apoptotic cells. *A*, DCs cultured as described are immature on day 7, as assessed by levels of expression of costimulatory molecules and MHC class II. Stimulation by LPS for 24 h induced a mature phenotype in a dose-dependent manner. DCs were prepared as described in *Materials and Methods* and stimulated with 0 (thin lines), 0.1 (dashed lines), or 0.5 μg/ml (thick lines) LPS for 24 h and assessed by flow cytometry. Isotype controls are omitted for clarity but sit in the first log order of fluorescence. Data shown are from one experiment, representative of at least five similar experiments. *B*, Immature DCs were incubated with fluorescently labeled apoptotic neutrophils at a ratio of 1:5. Flow cytometric assay of interaction between DCs and apoptotic cells demonstrating at 37°C green fluorescence of phagocytic DC, which was almost completely inhibited at 4°C. Events were gated for CD11c-positive cells and were representative of at least three experiments. *C*, DC were incubated with fluorescent apoptotic cells and counterstained with FITC-conjugated I-A^d/I-E^d (MHC II). By fluorescence microscopy, red fluorescent apoptotic bodies interact with MHC II-positive DCs (green) and, after internalization by a DC, change their fluorescent properties (arrow). Original magnification, ×63 oil. Scale bar represents 10 μm.

rates of phagocytosis were seen when dexamethasone-treated murine thymocytes were used as an alternative source of apoptotic cells (data not shown). Thus, immature murine DCs exhibited a capacity for phagocytosis of apoptotic cells similar in magnitude to that reported in studies of immature human DC (7, 8).

Ingestion of apoptotic cells specifically inhibits the ability of DCs to up-regulate CD86

To ascertain whether ingestion of apoptotic cells alters DC phenotype, cell surface expression of activation markers was studied by flow cytometry after phagocytosis. No significant difference in cell surface expression of the costimulatory molecules CD40, CD80, and CD86 was seen between immature DCs that had ingested apoptotic cells (ac^+) and those that had not (ac^-) either immediately (2 h, data not shown) or 24 h (Fig. 2) after phagocytosis, confirming that ingestion of apoptotic cells did not activate DCs. However, on DC maturation with LPS a marked difference in surface expression of CD86 was detected between ac^+ DCs and ac^- DCs. Immature DCs were predominantly $CD86^{low}$, with a small population of $CD86^{high}$ cells. Upon maturation driven by LPS the proportion of $CD86^{high}$ cells increased in a dose-dependent manner (Fig. 1*A*). However, fewer ac^+ DCs became $CD86^{high}$ compared with ac^- DCs; in a typical experiment at 0.1 μg/ml LPS only 13.2% of ac^+ DCs became $CD86^{high}$ vs 42% ac^- DCs, and this difference was maintained at the highest LPS dose of 0.5 μg/ml, with only 24% of ac^+ DCs becoming $CD86^{high}$ compared with 46% of ac^- DCs (Fig. 2). Similar results were seen when DCs were matured with SAC (data not shown). Mature DCs were heterogeneous for CD54 with distinct populations of $CD54^{low}$ and $CD54^{high}$ cells, becoming most apparent at the highest dose of LPS (0.5 μg/ml). Despite the general increase in fluorescent intensity of the ac^+ DCs after phagocytosis, it is apparent that the percentage of $CD54^{high}$ cells was lower in ac^+ than ac^- populations (Fig. 2). CD40 expression was unaffected by apoptotic cells, with maturation in response to 0.5 μg/ml generating a single $CD40^+$ population (Fig. 2), and no statistically significant difference was seen in MHC class II or CD80 expression (data not shown). Hence, the failure to up-regulate CD86 in the ac^+ DCs was not due to general unresponsiveness to LPS but appeared to affect a subset of costimulatory molecules.

Failure to up-regulate CD86 expression in ac^+ DCs could reflect preferential ingestion of apoptotic cells by a subpopulation of DCs destined not to become $CD86^{high}$ in response to maturation stimuli. However, when the mean CD86 fluorescence for the whole DC population was compared between DCs matured in the presence or the absence of apoptotic cells (Fig. 3, *A* and *B*), the presence of apoptotic cells resulted in significantly lower CD86 fluorescence for the whole DC population compared with the control. No such difference would have been detectable had the capacity to ingest apoptotic cells merely marked a subpopulation of DCs destined not to up-regulate CD86 in response to LPS. This and the observation that no significant difference in costimulatory molecule expression was detected between the ac^+ DCs and ac^- DC population immediately after ingestion (data not shown) support the fact that phagocytosis had not preferentially occurred in a subpopulation destined to be $CD86^{low}$. Also, this inhibitory effect was not merely a result of particle ingestion, as immature DCs, when cocultured with latex beads and oxidized lipid, showed remarkably high levels (>85%) of phagocytosis and endocytosis, respectively, and exhibited no defect in LPS-driven up-regulation of CD86 expression, exhibiting, instead, apparently enhanced expression (Fig. 3*C*). Taken together these data support the hypothesis that apoptotic cell

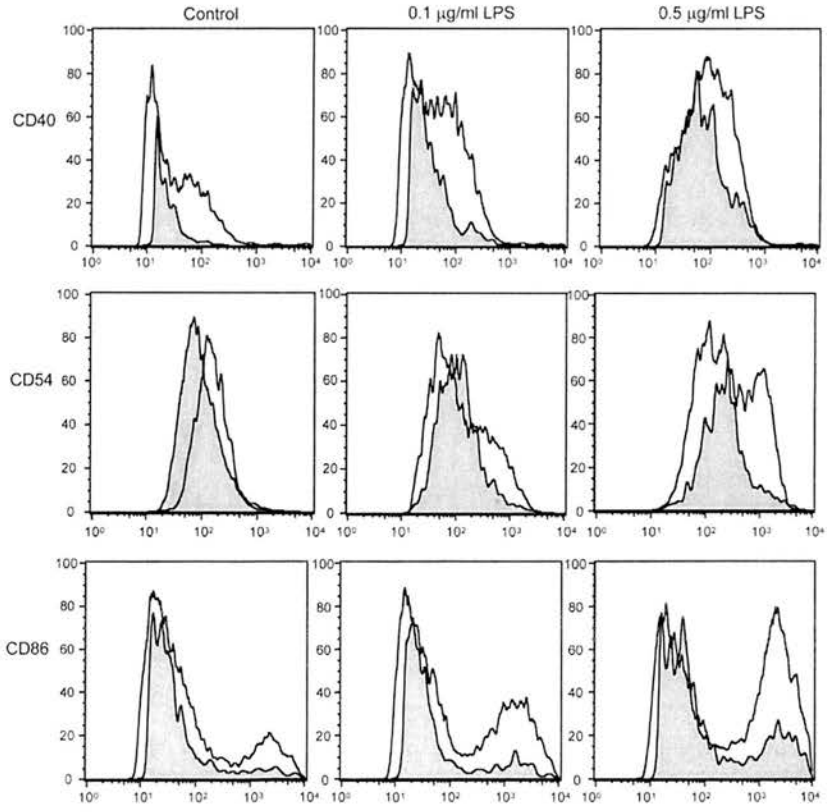

FIGURE 2. DCs that have internalized apoptotic cells, but not control particles, fail to up-regulate CD86 normally in response to LPS or SAC, but up-regulate other markers, such as CD40 and CD54. DCs were incubated with green fluorescent apoptotic cells, matured with LPS, and stained for surface costimulatory molecules. DCs were gated into ac^+ (filled histogram) and ac^- (open histogram) by incorporation of green fluorescence. Isotype control Abs are not shown for clarity but sat in the first log order of fluorescence intensity. Data are representative of three experiments.

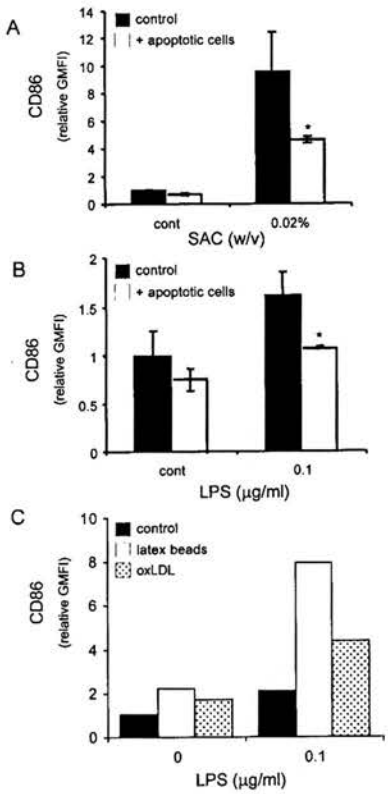

FIGURE 3. Surface CD86 expression of bulk DC cultures containing both ac^+ DCs and ac^- DCs was inhibited by apoptotic cells. DCs were incubated with apoptotic cells and subsequently matured with SAC (0.02%, w/v; *A*) or LPS (0.1 μg/ml; *B*). Surface CD86 expression was measured by FACS and is expressed as mean fluorescence relative to control unstimulated DCs. Relative fluorescence of DCs cocultured with apoptotic cells (□) or medium alone (■) is shown, demonstrating that apoptotic cells can suppress both LPS- and SAC-driven DC maturation. Apoptotic cells were excluded from FACS analysis on the basis of small size and bright green fluorescence. The mean fluorescence ± SD for triplicate measurements from one culture is shown. Similar results were seen in three (SAC) or five (LPS) independent experiments. *, $p < 0.05$ (by ANOVA). *C*, DCs were cultured with latex beads (□) or oxidized LDL (▨); compared with incubation with no particles (■), these control particles augmented, rather than decreased, CD86 expression. The mean fluorescence of duplicate cultures is shown, and similar results were seen in two independent experiments.

ingestion alters the subsequent response of DCs to maturation stimuli.

Ingestion of apoptotic cells modulates proinflammatory cytokine expression by DCs

Cytokines produced by DCs are especially important in determining subsequent T cell responses. We therefore examined the effect of ingestion of apoptotic cells on cytokine production by DCs by combining the fluorescent phagocytosis assay and intracellular cytokine staining of cells, allowing us to study the production of cytokines by individual DCs (Fig. 4). The autocrine response to TNF-α produced after LPS stimulation is an important factor in terminal maturation and activation of DCs as well as recruitment and activation of neighboring effector cells. Interestingly, virtually all the DCs containing apoptotic cells expressed TNF-α after stimulation with LPS for 5 h, demonstrating their functional viability and continuing responsiveness to LPS stimulation. A small population of the ac^- DCs failed to produce TNF-α and probably represented a population of fully matured or "exhausted" DCs (19) (Fig. 4). IL-12 is produced predominately by DCs and orchestrates both the innate and adaptive immune responses. DCs express a functional IL-12R, ligation of which by bioactive IL-12p70 augments LPS maturation. In contrast to TNF-α, ac^+ DCs failed to express IL-12 even when stimulated with 0.5 μg/ml LPS (Fig. 4). Similar results were seen when DCs were stimulated with SAC (data not shown).

Inhibitory effects of apoptotic cells on DCs are not mediated by autocrine/paracrine effects of IL-10 or TGF-β1

IL-10 is an important anti-inflammatory cytokine associated with induction of tolerance, resolution of inflammation, inhibition of production of proinflammatory cytokines, and DC maturation (20). IL-10 has been shown to inhibit DC maturation, acting in both a paracrine and an autocrine manner (21). Interaction of apoptotic cells with monocytes (22), but not macrophages (14), has been shown to induce the production of IL-10. To investigate whether changes in IL-10 expression by DCs ingesting apoptotic cells might contribute to the different phenotype, intracellular IL-10 production and release into the supernatant were studied. IL-10 was detectable in DC culture supernatant but was unaffected by interaction with apoptotic cells or the addition of LPS (Fig. 5*A*). Intracellular IL-10 was difficult to detect reliably over background staining, and no differences in levels of IL-10 staining between ac^+ and ac^- DCs were detectable (data not shown). Furthermore, blockade of functional IL-10 by soluble IL-10R did not differentially affect costimulatory molecule expression in the two DC subpopulations (data not shown). Finally, LPS activation of DCs derived from bone marrow of IL-10-deficient mice was also inhibited by the ingestion of apoptotic cells. Interestingly, these DCs demonstrated a heightened responsiveness to LPS, confirming an autocrine feedback role for IL-10 in DC maturation (Fig. 5*B*).

TGFβ-1 is another important inhibitory cytokine implicated in anti-inflammatory effects of apoptotic cells. TGF-β is found in apoptotic cells, preferentially localized to the mitochondria (23), as well as being secreted by macrophages ingesting apoptotic cells (14–16). Although TGF-β1 could be found in our culture supernatants, levels of serum contamination made determining its origin difficult (data not shown). However, when a soluble TGF-βR was used to neutralize active TGF-β1 released by DCs they were still inhibited after ingesting apoptotic cells (Fig. 5*C*). In contrast, soluble TGF-βR was capable of blocking inhibition of TNF-α production by macrophages that had ingested apoptotic cells in a parallel system (Fig. 5*D*). Taken together these data mitigate against a role for autocrine IL-10 or TGF-β1 in inhibiting the DC response to LPS while confirming the previously reported role for TGF-β in the inhibition of macrophages that have ingested apoptotic cells.

Ingestion of apoptotic cells generates DCs with diminished capacity to sustain Ag-dependent unprimed T cell proliferation despite LPS maturation

To determine whether these cytokine and surface CD86 differences reflected a distinct functional phenotype of DCs, we chose to examine the capacity of ac^- DCs and ac^+ DCs to sustain Ag-dependent naive T cell proliferation, a process critically dependent on IL-12 production and expression of costimulatory molecules. The use of unprimed T cells from DO11.10 TCR-transgenic mice allowed us directly to compare T cell proliferation in response to mature ac^+ DCs vs ac^- DCs, pulsed in both cases with $OVA_{323-339}$ peptide after LPS maturation. Interestingly, ac^+ DCs retained the ability to sustain naive T cell proliferation but were only ~30% as effective as stimulators compared with ac^- DCs or

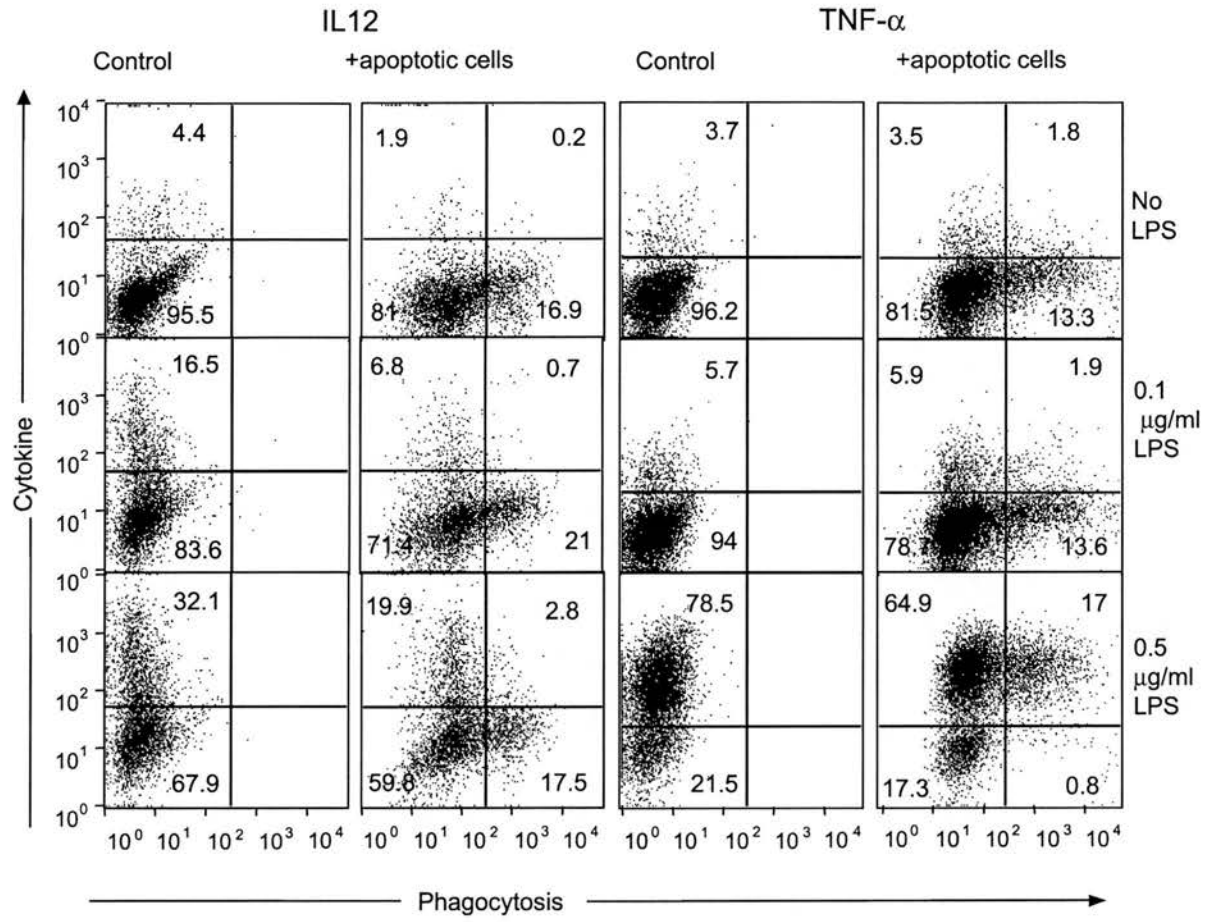

FIGURE 4. Apoptotic cells inhibit IL-12 production, but not TNF-α production, by LPS-stimulated DCs. DCs were incubated with green fluorescent apoptotic cells and stimulated with LPS. Intracellular cytokine production was measured after 4 h by FACS analysis gated on CD11c-positive cells (DCs). DCs that have ingested apoptotic cells can be distinguished by the incorporation of green fluorescence. The quadrant markers are set on isotype control Abs (cytokine) or DCs prepared without apoptotic cells (phagocytosis), and figures give the percentage of cells in each quadrant. The data are representative of three independent experiments.

DCs matured without apoptotic cells when cultured at a ratio of 10:1, T cells:DCs (Fig. 6).

Discussion

The data presented demonstrate that DC ingestion of apoptotic cells, but not control particles, results in subsequent down-regulation of LPS-driven IL-12 production and CD86 expression. Furthermore, this correlated with impaired Ag-dependent T cell activation in vitro. Interestingly, these effects were restricted to those DCs that had ingested apoptotic cells and were not due to anti-inflammatory cytokine production, implicating ligation of specific phagocytic receptors in this process.

Bone marrow-derived DCs and macrophages arise from common myeloid precursors and share many characteristics while maintaining subtle differences in responses and effector functions. Ingestion of apoptotic cells stimulates macrophages to adopt an anti-inflammatory phenotype, inhibiting LPS-induced release of TNF-α and up-regulating release of TGF-β1 and other anti-inflammatory mediators (24, 25). Furthermore, previous reports and our unpublished data emphasize that this phenotypic change in macrophages ingesting apoptotic cells is spread to surrounding cells through the paracrine action of cytokine release triggered by the ingestion of apoptotic cells. This is in contrast to the response of DCs ingesting apoptotic cells, which, in this current study, did not affect the ability of neighboring DCs that had not ingested apoptotic cells to mature or stimulate T cells. This would implicate a direct and cell-specific effect of apoptotic cell ingestion on DC expression of CD86 and cytokine production, rather than a paracrine effect of secreted anti-inflammatory cytokines. Our data lend weight to the possibility that the different recognition mechanisms employed for ingestion of apoptotic cells by monocyte-derived phagocytes might determine the different responses seen between macrophages and DCs (7, 26), although we have not set out to define the receptors for apoptotic cells implicated in the inhibition of DC maturation in this study. The preferential inhibition of IL-12 has been demonstrated by ligation of a large number of phagocytic receptors used by macrophages, including some that are also expressed on DCs (27, 28). The CD36/integrin $\alpha_v\beta_5$/thrombospondin complex has been implicated as the major receptor for apoptotic cells in DC phagocytosis, and recently binding of both malaria-infected erythrocytes (29) or apoptotic cells (30) to this complex has been shown to inhibit human DC maturation. Although these studies did not address whether apoptotic cell internalization must

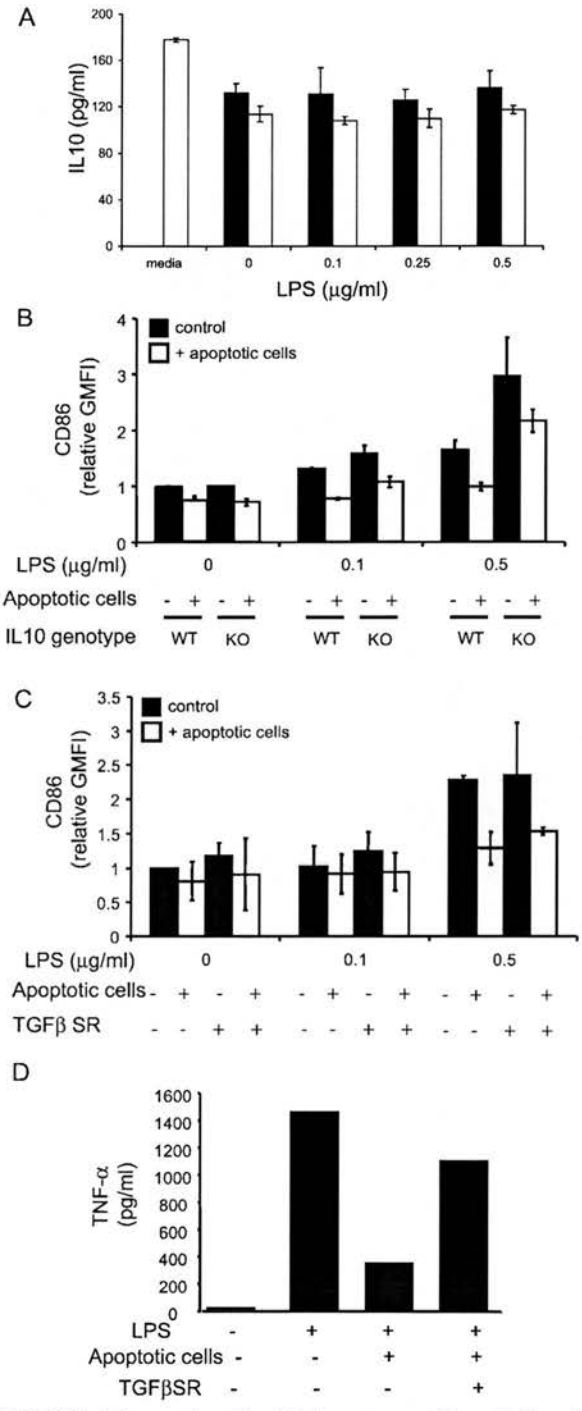

FIGURE 5. The secretion of anti-inflammatory cytokines, IL-10 and TGF-β, is not responsible for the observed changes in DC function. *A*, Apoptotic cells do not induce the release of IL-10 from DCs. Twenty-four-hour supernatants from control DCs (■) and DCs cocultured with apoptotic cells (□) stimulated with varying doses of LPS were assayed for IL-10 by ELISA. The mean ± SD of triplicate measurements from one culture supernatant is shown. Constitutive amounts of IL-10 detected in fresh medium containing hybridoma supernatant is shown (media) as baseline. Similar results were seen in three independent experiments. *B*, DCs derived from bone marrow of IL-$10^{-/-}$ mice were also inhibited by apoptotic cells. WT DCs with (□) and without (■) apoptotic cells demonstrate inhibition of CD86 expression. Similar results were seen when IL-$10^{-/-}$ DCs were incubated with or without apoptotic cells. Note the generally higher levels of expression of CD86 in IL-10-deficient DCs. Data are the mean ± SD from triplicate wells from one knockout or wild-type mouse representative of four similar mice. The presence of TGF-β soluble receptor (*C*) does not affect the inhibitory capacity of apoptotic cells. DCs were cultured with apoptotic cells as described in *Materials and Methods* in the presence or the absence of TGF-β soluble receptor. Data are the mean ± SD from triplicate wells from one culture. Similar results were seen in two independent experiments. *D*, TGF-β soluble receptor does inhibit the effect of apoptotic cell ingestion on TNF-α production by LPS-stimulated mouse bone marrow-derived macrophages. Macrophages were incubated with LPS (1 μg/ml) and/or apoptotic cells, and TNF-α production was measured by ELISA after 24 h.

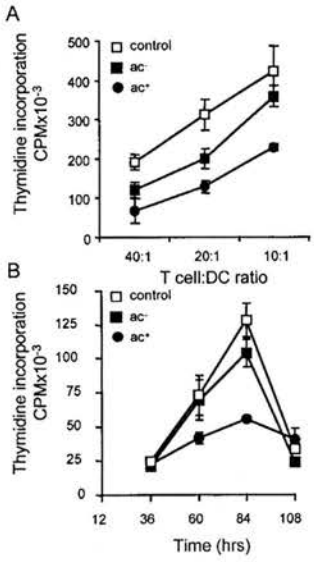

FIGURE 6. Ag-driven T cell stimulation was diminished in DCs that ingested apoptotic cells despite LPS stimulation. Mature DCs were pulsed with $OVA_{323-339}$ peptide, sorted into ac^- (■) and ac^+ (●) cells, and incubated with T cells from DO11.10 mice. DCs that had not been incubated with apoptotic cells are included as a control (□). T cell proliferation was measured by [^{3}H]thymidine incorporation. *A*, Proliferation in response to different T cell:DC ratios, measured after 3 days of interaction. *B*, Time course of T cell/DC interaction demonstrated inhibition at all time points. Background proliferation in the absence of OVA peptide was <2000 cpm in all experiments. The mean ± SD from triplicate measurements in one experiment, representative of three independent experiments, are shown.

occur, our data imply that a direct interaction is necessary for these effects to be seen.

Apoptotic cells are poorly immunogenic and, unless they overload normal clearance mechanisms (probably becoming secondarily necrotic) or are associated with danger signals, rarely incite an immune response (31). Furthermore, UV irradiation, characterized by widespread apoptosis, is associated with generalized immunological hyporesponsiveness, demonstrating a potential immunosuppressive effect of apoptotic cells on the adaptive immune system. In an interesting recent report, injection of apoptotic cells was able to promote bone marrow engraftment even across MHC barriers in a species-independent manner (32). Although the exact mechanisms of immunosuppression in such systems are not fully understood, a bone marrow-derived cell, likely to be the DC, has been implicated. Exactly how this occurs is controversial, but an increasingly accepted view is that the immature DC, with low levels of costimulatory molecule expression, would fail to deliver

signal 2 and induce anergy or deletion of an interacting T cell. In support of this, repeated immunization with immature DCs does appear to induce Treg/Tr1 cells. In contrast, mature DCs, which are able to secrete a potent stimulatory cytokine, IL-12, and express high levels of costimulatory molecule expression, induce strong adaptive immunity. IL-12 has been shown to have a myriad of functions, including modulating Th1 vs Th2 switching, activation of NK cells, and production of IFN-γ. Furthermore, the autocrine effects of IL-12 have been shown to augment DC responses to exogenous stimuli, underscoring the importance of this cytokine in DC effector functions and the subsequent adaptive immune response. Although failure of ac^+ DCs to produce high levels of IL-12 might contribute to some of the subsequent phenotypic changes, including diminished T cell stimulation, we have no direct evidence of whether this is indeed the case and is the subject of ongoing investigation. Nevertheless, failure to produce IL-12 after apoptotic cell ingestion appears to correlate closely with these changes.

The importance of ingestion of apoptotic cells by DCs is underscored by circumstantial evidence implicating such DCs in maintaining tolerance. For example, a specific population of rat lymph DCs, characterized by being $OX41^-CD4^-$, has been described. These cells represent a major population found in the lymph draining the intestinal epithelium and have blunt pseudopodia and coarse granular inclusions, identified as being derived from apoptotic intestinal epithelial cells. Functionally, these $OX41^-CD4^-$ rat lymph DCs also demonstrate an impaired ability to stimulate T cells in vitro and have been implicated in the ability of the gut to handle large amounts of foreign Ags in a tolerogenic fashion (33–35). Interestingly, our in vitro cultured ac^+ DCs also share some of these physical characteristics with the $OX41^-CD4^-$ rat lymph DCs (our personal observations). While comparisons between in vivo/ex vivo studies of rat DCs and our in vitro work on murine DCs need to be made with caution, taken together these data lend strong support to the concept that ingestion of apoptotic cells by DCs modulates their function.

In conclusion, it is essential for DCs to mature before they can activate naive T cells, and our data and two recent studies (4, 5) confirm that ingestion of apoptotic cells alone did not provide sufficient maturation stimulus. However, some necrotic cells or virally infected apoptotic cells are effective stimulators of DC maturation. Therefore, DCs ingesting apoptotic cells must be exposed to additional agents, such as necrotic cells, monocyte-conditioned medium, or viral products, before they become capable of stimulating T cells. Many of these agents will be present in inflamed sites alongside apoptotic cells in vivo, and the potential for DCs both to acquire apoptotic cell-derived self-Ags and receive maturation signals is high. However autoimmunity is uncommon, and the response of the DC is likely to be tightly regulated. We suggest that ingestion of apoptotic cells is not immunologically null, but is capable of regulating DC maturation, providing a counterbalance for inflammatory stimuli. A failure to see these inhibitory effects of apoptotic cells in other studies may reflect the percentage of DCs ingesting apoptotic cells and the strength of the maturation stimulus used. In the future, defining whether apoptotic cells themselves are sufficient to alter DCs effector functions in vivo will be of great interest. Further investigating this process will increase our understanding of the mechanisms controlling peripheral self-tolerance while giving us new insights into strategies for Ag delivery that might generate tolerance rather than immunity. In contrast, understanding how apoptotic tumor cells or pathogens might also use this phenomenon for immune evasion will increase our understanding of tumor immunology and infectious disease.

Acknowledgments

We thank Prof. N. A. Mitchinson and Dr. E. Lightstone for their helpful discussion, and S. MacCall and M. Clay for their technical support.

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Nonphlogistic Clearance of Late Apoptotic Neutrophils by Macrophages: Efficient Phagocytosis Independent of β_2 Integrins[1]

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Neutrophils undergo constitutive death by apoptosis, leading to safe nonphlogistic phagocytosis and clearance by macrophages. Recent work has shown that before secondary necrosis, neutrophils exhibiting classical features of apoptosis can progress to a morphologically defined late apoptotic state. However, whether such neutrophils could be safely cleared was unknown. We now report that human late apoptotic neutrophils could be purified from cultured neutrophil populations undergoing constitutive death and were subsequently ingested by human monocyte-derived macrophages by serum-independent mechanisms that did not trigger the release of IL-8 or TNF-α. Such ingestion was specifically inhibited by Abs to thrombospondin-1 and the $\alpha_v\beta_3$ vitronectin receptor. Murine bone marrow-derived macrophage phagocytosis of late and early apoptotic neutrophils occurred by similar mechanisms, proceeding with the same efficiency as that observed for wild-type controls when macrophages from $\alpha_m^{-/-}$ or $\beta_2^{-/-}$ mice were used. We conclude that specific nonphlogistic, β_2 integrin-independent mechanisms involving thrombospondin-1 and $\alpha_v\beta_3$ allow macrophages to ingest late apoptotic neutrophils without eliciting inflammatory cytokine secretion. *The Journal of Immunology,* **2001, 166: 4743–4750.**

Neutrophils and their toxic contents are vital for host defense, but may also mediate undesirable tissue injury in a wide range of inflammatory diseases (1, 2). However, a growing body of evidence indicates that neutrophils can be safely eliminated from inflamed tissues, promoting resolution of the inflammatory response, by constitutively undergoing apoptosis (3–9). This is a programmed form of cell death that leads to swift phagocyte recognition, uptake, and degradation of intact senescent neutrophils, preventing leakage of noxious contents from the dying cells and failing to elicit proinflammatory responses from the ingesting phagocyte (10–13). Consequently, safe phagocytic clearance of neutrophils undergoing constitutive apoptosis is viewed as a key control point in the inflammatory response.

However, the molecular mechanisms mediating safe phagocytic clearance of apoptotic cells remain poorly understood; an increasing number of phagocyte receptors (see Refs. 14–16 for reviews) have been implicated in vitro. This complexity may reflect the fact that studies have frequently involved administration to phagocytes of "meals" consisting of heterogeneous populations containing cells at various stages of the death program, including secondary necrosis (17). By contrast, human neutrophils undergoing constitutive apoptosis during overnight culture contain a mixture of histologically normal neutrophils (not ingested by phagocytes) and intact cells that exclude trypan blue and propidium iodide (PI)[3] and exhibit classical morphologic features of early apoptosis (3). These include nuclear coalescence and chromatin condensation accompanied by well-defined surface changes such as the capacity to bind annexin V (18–20). By separating intact apoptotic neutrophils from histologically normal neutrophils within the same population of aging cells, we confirmed that phagocytes recognized only the apoptotic neutrophils (3). Nevertheless, even this apparently "clean" model system has proved more complex than was first thought. Beyond 18 h in culture, a steadily increasing proportion of senescent neutrophils exhibit a characteristic late apoptotic morphology in which nuclear degradation or so-called evanescence is accompanied by electron microscopic evidence of limited granule fusion with the plasma membrane (21, 22). To avoid confusion, we propose that neutrophils with classical features of apoptosis (3) should be regarded as early apoptotic cells.

We have become interested in whether late apoptotic neutrophils are recognized by macrophages (Mϕs) and, if so, the molecular mechanisms and consequences of this event. A number of different types of phagocytes can deploy the phagocyte surface $\alpha_v\beta_3$ vitronectin receptor integrin to present bridging thrombospondin 1 (TSP1) to apoptotic cells and promote phagocytosis without inciting proinflammatory secretory responses (10, 11, 23, 24). This mechanism was a strong candidate for Mϕ recognition of late apoptotic neutrophils, because 1) these cells exhibit limited fusion with the plasma membrane of granules containing proteins

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Received for publication October 11, 2000. Accepted for publication January 30, 2001.

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[1] This work was supported by the Wellcome Trust (Grant 047273). Y.R. was a Dorothy Hodgkin Fellow of the Royal Society, and F.L. is supported by the Howard Hughes Medical Institute and the Arthritis Foundation.

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[3] Abbreviations used in this paper: PI, propidium iodide; TSP1, thrombospondin 1; CR3/4, complement receptor type 3/type 4; Mϕ, macrophage; MPO, myeloperoxidase; PMN, polymorphonuclear neutrophil granulocyte; PPP, platelet-poor plasma; PRPDS, platelet-rich plasma-derived serum; RGDS, Arg-Gly-Asp-Ser; RGES, Arg-Gly-Glu-Ser.

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capable of binding TSP1 (21, 22, 25); 2) a very recent report indicates that late apoptotic neutrophils bind TSP1 with such efficiency that this can be demonstrated with soluble biotinylated TSP1 (19); and 3) myeloid dendritic cells bind late apoptotic cells via an α_v-mediated mechanism (26). However, equally strong candidate phagocyte receptors were those of the β_2 integrin family that bind opsonic complement fragments, type 3 complement receptor (CR3; $\alpha_m\beta_2$ or CD11b/CD18) and CR4 ($\alpha_x\beta_2$ or CD11c/CD18). Not only does ligation of these receptors fail to stimulate the release of inflammatory mediators from Mϕs (27, 28), but there is also evidence that they can bind, via opsonic complement fragments, populations of dying cells (17, 29). Furthermore, β_2 integrins can also bind denatured proteins (30), which might be exposed by late apoptotic cells.

Therefore, in this study we set out to determine whether it was possible to purify late apoptotic neutrophils from cultured neutrophil populations undergoing constitutive death, whether such cells were nonphlogistically ingested by Mϕs, and whether either Mϕ TSP1/$\alpha_v\beta_3$ or β_2 integrins mediated phagocytosis of late apoptotic neutrophils.

Materials and Methods

Tissue culture materials

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Culture media (HBSS, IMEM, and DMEM) and supplements (100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% FCS) were obtained from Life Technologies (Grand Island, NY). Sterile tissue culture plasticware was purchased from Falcon Plastics (Cockeysville, MD).

Abs and peptides

mAb to the human vitronectin receptor integrin (31) was the $\alpha_v\beta_3$-specific mAb 23C6 (IgG1; provided by Prof. M. Horton, St. Bartholomew's Medical School, London, U.K.). V. Dixit (University of Michigan, Ann Arbor, MI) provided mTSP1, a rabbit affinity-purified polyclonal Ab specific for mouse TSP1 (32), and A6.1, an IgG1 murine mAb for human TSP1 (33). H9.2B8, a hamster mAb specific for the mouse integrin α_v chain (34) was obtained from PharMingen (San Diego, CA). The control mAb was mAb OX-7 (from Serotec, Banbury, Oxon, U.K.), an IgG1 that recognized Thy 1.1. The tetrapeptides Arg-Gly-Asp-Ser (RGDS) and Arg-Gly-Glu-Ser (RGES) were obtained from Sigma.

Human leukocytes

Neutrophils were isolated from fresh citrated normal human blood by dextran sedimentation and plasma-Percoll discontinuous density gradient centrifugation and were "aged" in tissue culture in IMEM with 10% autologous platelet-rich plasma-derived serum (PRPDS) so as to undergo apoptosis, exactly as previously described (3). Late apoptotic neutrophils were purified from 22-h aged polymorphonuclear neutrophil granulocyte (PMN) by discontinuous plasma-Percoll density gradient centrifugation; as described later in this report, >95% pure late apoptotic cells were isolated at the interface between platelet-poor plasma (PPP) and 31% Percoll in PPP, whereas a mixed population of late apoptotic and early apoptotic cells was found at the interface of 31 and 42% Percoll in PPP; the latter was not used in phagocytosis assays. A mixed population of early apoptotic and nonapoptotic neutrophils free of late apoptotic cells was retrieved at the 42–51% Percoll in PPP interface; this last population was used as early apoptotic neutrophils in studies of recognition by Mϕ (see below), as all our previous studies have used similar mixed populations that routinely arise after overnight aging in culture, because such cells are >99% viable by trypan blue exclusion (3, 6, 23, 24). Gradients were centrifuged at 610 × *g* for 24 min at 4°C. Human monocytes (>90% pure) were prepared by counterflow centrifugation as described previously (35) and cultured for 5 days in IMEM with 10% autologous prpds to mature into Mϕ as previously described (35).

Isolation and culture of mouse Mϕ

Bone marrow was harvested from BALB/c mice and plated in 96-well plates in DMEM containing 10% FCS and 10% L929 cell-conditioned medium as a source of M-CSF. Bone marrow Mϕ were used in phagocytosis assays after 7 days of growth in culture as previously described (34).

In some experiments Mϕ were prepared as described above from gene-targeted mice and wild-type strain controls as follows: α_m $(CD11b)^{-/-}$ BALB/c mice and BALB/c controls were used as previously described (36); β_2 (CD18) null mice were crossed from 129sv/eg onto the C57BL/6J background, and F_2 $CD18^{-/-}$ animals were used as founders for the $CD18^{-/-}$ mice used in the current experiments, with control mice bred from 129sv/eg and C57BL/6J in parallel (37).

Assessment of PMN morphology and flow cytometric analysis

Early apoptotic and late apoptotic PMN were assessed by microscopic examination of cytocentrifuge preparations fixed in methanol and stained with May-Giemsa or by transmission electron microscopy as previously described (3). Binding of FITC-conjugated annexin V in ice-cold PBS containing calcium and magnesium as previously described (18, 20) was used to assess exposure of phosphatidylserine exposure. PI was used to assess plasma membrane permeability. Cells were exposed to PI at 1 μg/ml for 120 s immediately before flow cytometric analysis; a positive control was provided by brief heating of 22-h aged neutrophil populations to 100°C. Labeled cells were applied to a Becton Dickinson FACScan flow cytometer (Mountain View, CA) that automatically and simultaneously measured the fluorescence of individual cells identified by their size-dependent light-scattering properties.

Interaction assay

A coded, observer-blind, microscopically quantified phagocytic assay of Mϕ ingestion of apoptotic PMNs, which has been extensively described, illustrated, and validated (3, 23, 24, 38), was used in these studies. Apoptotic and late apoptotic PMNs prepared as described above from a single population of 22-h aged PMNs were washed once in HBSS and suspended in IMEM, and 0.5 × 10^6 PMN in 50 μl of medium were added to each washed well of Mϕ cultured in 96-well plates. After interaction for 30 min at 37°C in 5% CO_2, the wells were washed in cold (4°C) 0.9% saline to remove noningested PMNs, and then the Mϕ monolayer was fixed in 2% glutaraldehyde in saline for 2 min and stained for myeloperoxidase (MPO), and the proportion of Mϕ-ingesting PMNs was counted by inverted light microscopy, exactly as previously described (3, 23, 24, 38). Because of a tendency of nonapoptotic neutrophils to adhere to mouse bone marrow Mϕ after 30-min interaction, mouse Mϕ were then trypsinized, and a separate cytocentrifuge preparation was prepared for each well as previously described (11, 35). These were fixed with 2% glutaraldehyde, stained for MPO, and finally counterstained with Hemalum (BDH, Poole, U.K.). The proportion of mouse Mϕ-containing, brown-staining, MPO-positive PMNs was then counted.

Effects of serum

To determine whether there were effects of serum on the phagocytosis of early apoptotic and late apoptotic PMN, such PMNs were interacted with human monocyte-derived Mϕ in the presence of 15% normal nonheated autologous human PRPDS or serum prepared in glass and 15% normal heated (56°C for 30 min) autologous human PRPDS or serum from glass.

Effects of Abs

These were determined as previously described (23, 24, 34, 38). Mϕ in 96-well plates were washed, and 50 μl of Ab at the desired concentration in IMEM was added to each well. The plates were incubated for 15 min at 4°C, followed by addition of 0.5 × 10^6 PMN in 10 μl of IMEM at 37°C, and then interacted for 30 min under standard conditions.

Effects of soluble inhibitors

Various inhibitors were included in the interaction medium. The tetrapeptide RGDS and the control peptide RGES were made up in IMEM before being added to the interaction medium to achieve the desired concentration (1 mM).

Assessment of Mϕ response to ingestion of late apoptotic cells

Human Mϕ were washed with HBSS, then incubated with apoptotic PMN, late apoptotic PMN, and control particles in IMEM for 30 min. The noningested apoptotic PMN and control particles were washed away, and

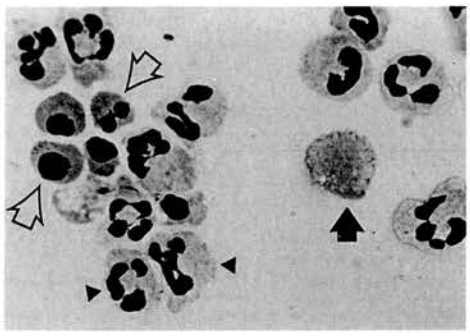

FIGURE 1. Heterogeneity of neutrophils aged in culture. Light microscopy (×1000) of cytoprep of 22-h aged neutrophils showing nonapoptotic neutrophils (examples marked by ▲), early apoptotic neutrophils (examples marked by open arrows), and a late apoptotic neutrophil (closed arrow). Note the lack of chromatin in the latter.

IMEM was added for 16 h. Supernatants were collected at this time point because cytokine secretion stimulated by opsonized zymosan was maximal (13). Supernatants were centrifuged at 6000 rpm for 3 min to remove particulate debris. Cytokine concentrations in the culture supernatants were assayed by ELISA for TNF-α and IL-8 using specific assays as previously described (11).

Results

Purification of late apoptotic neutrophils

In keeping with the studies by Hébert et al. (21), we observed increasing proportions of apparently anucleate late apoptotic neutrophils after 18 h of culture of normal human peripheral blood neutrophils (Figs. 1 and 2). We reasoned that it might be possible to purify late apoptotic neutrophils by density centrifugation on discontinuous Percoll-plasma gradients. From neutrophil populations aged in vitro for 22 h, we were able to obtain a fraction from the 0/31% Percoll interface containing >95% pure late apoptotic cells (Fig. 3*A*) with typical nuclear evanescence (21) and other characteristic features confirmed by electron microscopy (Fig. 3*B*) and a distinct fraction of aged neutrophils from the 42/51% Percoll interface displaying a mix of morphologically normal and early apoptotic neutrophils (Fig. 3*C*) closely similar to the overnight-aged neutrophil populations used in our previous studies of phagocyte recognition of early apoptotic neutrophils (23, 24, 34, 38). Typically around 50% of cells in this fraction displayed early apoptotic morphology (Fig. 3*C*; see also Fig. 4*B*).

When the phenotype of purified late apoptotic neutrophils from the 0/31% Percoll interface was further examined by flow cytometry, nonfixed cells stained with FITC-labeled annexin V, indicating exposure of phosphatidylserine (Fig. 4*A*) to a degree similar to that exhibited by the 50% of early apoptotic cells in the fraction from the 42/51% Percoll interface (Fig. 4*B*, *lower right quadrant*). The late apoptotic neutrophil population exhibited slightly greater permeability to PI than early apoptotic cells (compare Fig. 4, *A* and *B*), although much less than that exhibited by heat-permeabilized 22-h aged neutrophils (Fig. 4*C*). These data indicated that late apoptotic neutrophils exhibited some increase in plasma membrane permeability despite being morphologically intact. However, the late apoptotic neutrophil fraction isolated from 22-h aged neutrophil populations did not begin to undergo secondary necrosis, as evidenced by detectable spontaneous release of the granule constituent MPO, until recultured under standard conditions for at least 4 h (data not shown).

Human monocyte-derived Mϕ phagocytosis of late apoptotic cells is nonphlogistic

Previously we (10, 11, 39) and others (13) reported that uptake of apoptotic cells did not trigger release of proinflammatory cytokines from Mϕs and other phagocytes. However, Mϕ did release such cytokines when ingesting debris from granulocytes that had undergone secondary necrosis after constitutive apoptosis (39). Consequently, it was important to determine the response made by Mϕ taking up late apoptotic neutrophils. Following a 30-min interaction with late apoptotic neutrophils as described in *Materials and Methods*, 40.2 ± 1.8% (mean ± SE; $n = 6$) of human monocyte-derived Mϕ ingested late apoptotic neutrophils. However, by contrast with uptake of opsonized zymosan, there was no release of the proinflammatory cytokines, IL-8 or TNF-α, indicating that uptake of late apoptotic neutrophils was also nonphlogistic (Table I).

Human Mϕ phagocytosis of late apoptotic cells is not modulated by serum, but is inhibited by Abs to TSP1 and $\alpha_v\beta_3$

Although our previous studies have routinely used human neutrophils and Mϕ cultured in autologous serum obtained by recalcifying platelet-rich plasma (PRPDS), we have routinely washed both cell types before interaction in the absence of added serum (3, 23, 24, 35, 38). A recent report, using an assay of aged neutrophil interaction with human Mϕ in which much of the interaction signal appeared to be tethering of apoptotic cells rather than phagocytosis, suggested that interaction could be markedly enhanced by the presence of up to 15% serum as a source of complement (29). Nevertheless, the inclusion of 15% autologous serum prepared in glass did not enhance human monocyte-derived phagocytosis of either late apoptotic or early apoptotic neutrophil fractions in our assay (Fig. 5). Furthermore, the use of 15% PRPDS also failed to

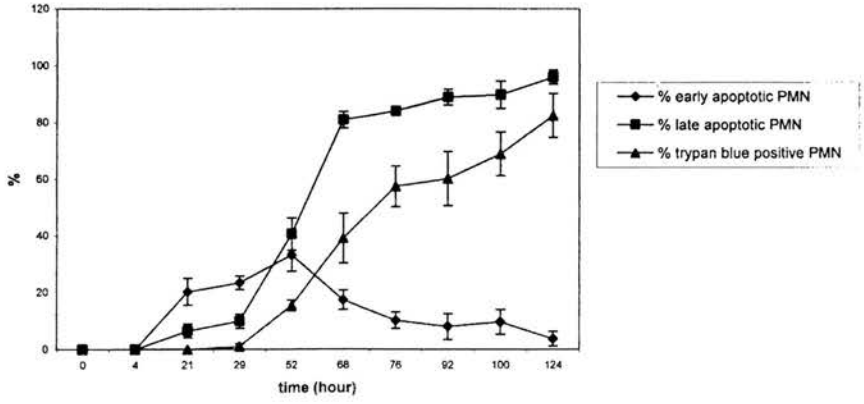

FIGURE 2. Time course of appearance of late apoptotic neutrophils during prolonged culture of purified neutrophils. With increasing time in culture note the successive appearance of early apoptotic cells (♦), late apoptotic cells (■), and intact cells admitting trypan blue dye (▲). Data are the mean ± SE ($n = 4$).

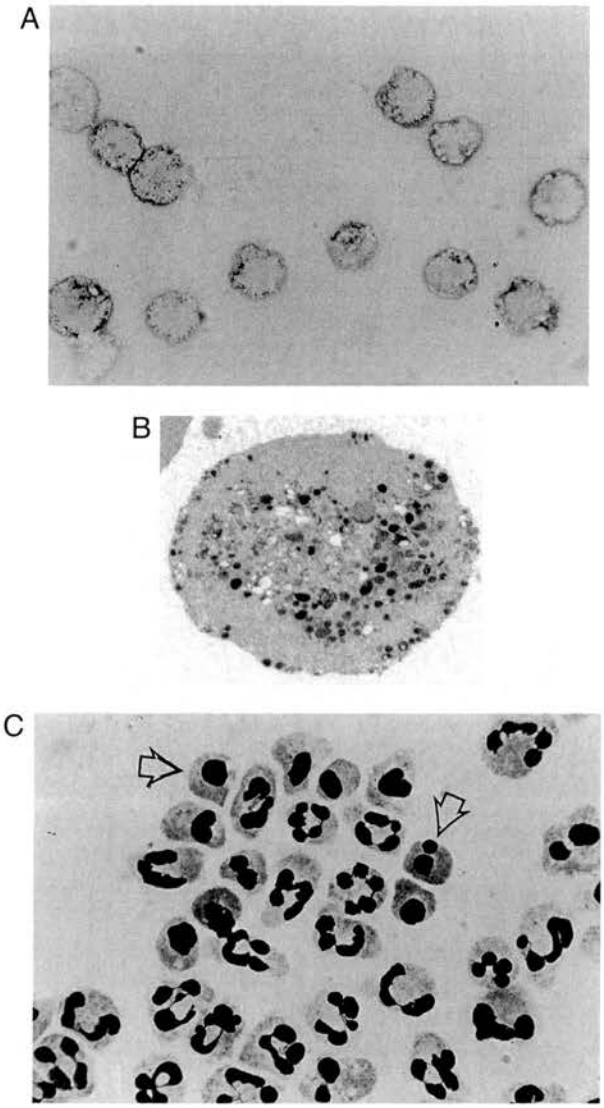

FIGURE 3. Purification of late apoptotic neutrophils. Human PMNs aged for 22 h in culture were separated on a discontinuous Percoll-plasma gradient and examined by light microscopy of May-Griemsa-stained cytopreps. *A*, Cells from the 0/31% Percoll interface; note that these senescent neutrophils have late apoptotic morphology. *B*, Transmission electron microscopy of a late apoptotic neutrophil (×10,000) prepared by plasma-Percoll density gradient centrifugation of 28-h aged cell population; note intact plasma membrane and retained granules. *C*, Cells from the 42/51% interface; note that this is a mixed population of nonapoptotic neutrophils and apoptotic neutrophils (examples; open arrows).

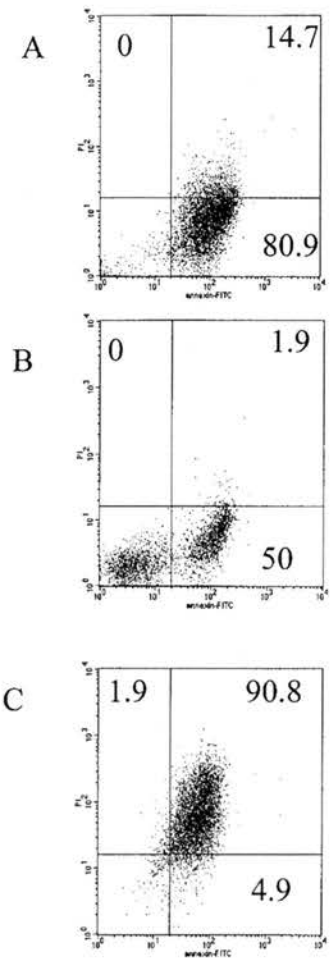

FIGURE 4. Permeability to PI and expression of phosphatidylserine by late apoptotic, early apoptotic, and heat-permeabilized aged neutrophils. Typical flow cytometric profiles for staining with FITC-annexin V (horizontal axis) and PI (vertical axis), comparing late apoptotic neutrophils (*A*) isolated from the 0/31% Percoll interface of discontinuous plasma-Percoll density gradient centrifugation of 22-h aged neutrophils, early apoptotic neutrophils (*right quadrants* of *B*) and normal neutrophils (*left quadrants* of *B*) isolated from the 42/51% Percoll interface, and heat-permeabilized 22-h aged neutrophils (*C*). Numbers in *upper left*, *upper right*, and *lower right quadrants* are the percentage of total cells in each quadrant. Note that >95% of late apoptotic neutrophils bind annexin V, and that such cells exhibit slightly increased PI staining compared with early apoptotic neutrophils.

enhance phagocytosis of each cell type (data not shown). By contrast, mAbs (but control mAb) to TSP1 and $\alpha_v\beta_3$ significantly inhibited human monocyte-derived Mϕ phagocytosis of both late and early apoptotic neutrophil fractions (Fig. 6), but not uptake of opsonized RBC used as controls (data not shown for clarity).

Murine bone marrow-derived Mϕ phagocytosis of late apoptotic neutrophils is also inhibited by Abs to TSP1 and α_v

Our previous work suggested that murine bone marrow-derived Mϕ use mechanisms similar to those of human monocyte-derived Mϕ in uptake of human early apoptotic neutrophils; inhibition by

Table I. *Proinflammatory cytokine release by human monocyte-derived Mϕ phagocytosing aged PMN*[a]

Stimulus	TNF-α (ng/ml)	IL-8 (ng/ml)
Control	2.60 ± 0.36	8.33 ± 1.35
Opsonised zymosan	589.29 ± 1.32*	62.75 ± 9.42*
Early apoptotic PMN	2.81 ± 1.49	4.81 ± 0.99
Late apoptotic PMN	1.61 ± 0.61	5.90 ± 1.45

[a] Note that phagocytosis of neither early apoptotic PMN by 38.3 ± 3.0% of Mϕ nor phagocytosis of late apoptotic PMN by 48.4 ± 1.9% of Mϕ stimulated cytokine release, by contrast with positive control stimulus of opsonised zymosan. Date are mean ± SE, $n = 4$; the only significant differences from control observed were in cytokine release after opsonized zymosan (*, $p < 0.001$).

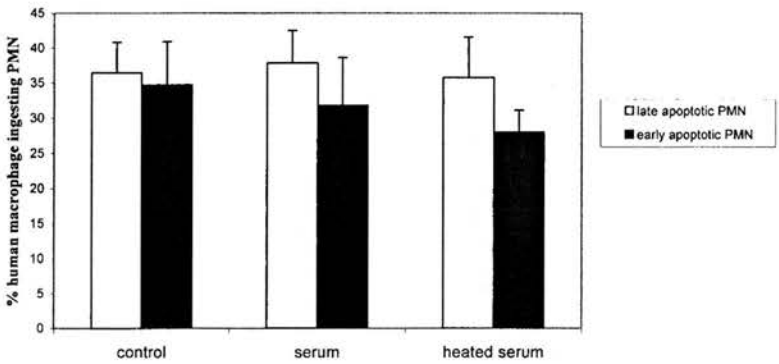

FIGURE 5. Serum does not potentiate phagocytosis of early or late apoptotic neutrophils. The presence of 15% autologous serum prepared by clotting blood in glass (bars in *center*) or decomplemented serum (heated at 56°C for 30 min; bars on *right*) did not potentiate phagocytosis of early apoptotic (■) or late apoptotic (□) by human monocyte-derived Mϕs compared with control conditions in which no serum was added to washed cells (bars on *left*). Values are the mean ± SE ($n = 6$). $p > 0.05$ in all cases.

RGDS peptide and anti-murine α_v mAb is demonstrable (34). Not only did these reagents inhibit uptake of late apoptotic neutrophils by murine bone marrow-derived Mϕ (but not control RGES peptide or control mAb; Fig. 7), but inhibition was observed with an affinity-purified rabbit Ab to TSP1 (but not by control rabbit IgG). No reagent used inhibited uptake of opsonized erythrocytes (data not shown for clarity).

β_2 integrins are not necessary for efficient Mϕ uptake of early and late apoptotic neutrophils

Because ligation of Mϕ CR3 and CR3-mediated uptake of particles have been reported not to activate proinflammatory responses from Mϕ (27, 28), we considered it important to examine the role of α_2 integrins in Mϕ uptake of early and late apoptotic neutrophils, particularly the $\alpha_m\beta_2$/CD11b CD18 CR3 integrin. Consequently, we sought definitive evidence of a requirement for $\alpha_m\beta_2$ or other β_2 integrins by studying Mϕ from gene-targeted mice. In experiments using bone marrow-derived Mϕ from $\alpha_m^{-/-}$ mice and wild-type controls, no difference was observed in the proportion of Mϕ ingesting late apoptotic neutrophils; these were highly efficiently ingested by 71.8 ± 5.3% of $\alpha_m^{-/-}$ Mϕ and 66.7 ± 5.9% of wild-type Mϕ (mean ± SE; $n = 3$; i.e., three mice in eachgroup; experiments were also performed in triplicate in Mϕ from each mouse). These findings were confirmed in a more extensive series of experiments using bone marrow-derived Mϕ from $\beta_2^{-/-}$ and wild-type control mice; there was no difference in the proportion of Mϕ ingesting either apoptotic or late apoptotic neutrophils (Fig. 8). Indeed, although recognition of late apoptotic neutrophils was generally slightly greater in absolute degree than uptake of early apoptotic cells in each set of experiments, perhaps reflecting unavoidable dilution of early apoptotic cells by copurified nonapoptotic neutrophils, the mechanism of recognition by bone marrow-derived Mϕ from wild-type or $\beta_2^{-/-}$ mice appeared very similar despite variation in the baseline degree of phagocytosis between sets of experiments consistent with previous experience (11, 34). Thus, specific inhibition of bone marrow-derived Mϕ uptake of late apoptotic neutrophils was observed with RGDS peptide at 1 mM, but not control RGES peptide, and was seen also with Ab to murine vitronectin receptor (α_v), but not control Ab (Fig. 8).

Discussion

Although previous work (21) has demonstrated that neutrophils undergoing constitutive death by apoptosis can pass from the early apoptotic state (as defined in our earlier studies (3)) to a late apoptotic state for some hours before finally undergoing secondary necrosis, it has been unknown whether late apoptotic neutrophils can be safely cleared by Mϕs. In this report we have demonstrated that fractions of morphologically defined late apoptotic neutrophils can be purified from populations of senescent human neutrophils. Furthermore, in keeping with previous data obtained with populations of 'early' apoptotic neutrophils (10, 11), late apoptotic neutrophils were efficiently ingested by human monocyte-derived Mϕ without triggering release of the pro-inflammatory mediators IL-8 and TNF-α. By contrast with a previous report (29), the proportion of human monocyte-derived Mϕ ingesting early apoptotic neutrophils was not increased by the presence of serum and no increase in uptake of late apoptotic neutrophils was observed. These data argued against a role for Mϕ β_2 integrins in uptake of dying neutrophils. However, the uptake of late apoptotic neutrophils by both human monocyte-derived Mϕ and murine bone marrow-derived Mϕ was inhibited by Abs to TSP1 and the vitronectin receptor, as

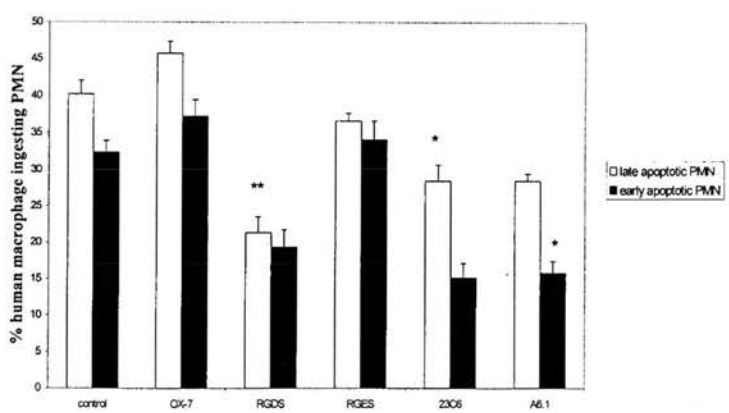

FIGURE 6. Human monocyte-derived Mϕ phagocytosis of aged PMN. Comparison of phagocytosis of late apoptotic PMNs (□) and early apoptotic PMNs (■) prepared from the same 22-h aged human PMN populations. Note that RGDS peptide, $\alpha_v\beta_3$ mAb 23C6, and TSP1 mAb A6.1 all inhibited phagocytosis of both types of aged PMN. Values are the mean ± SE ($n = 6$). =, $p < 0.05$; **, $p < 0.001$.

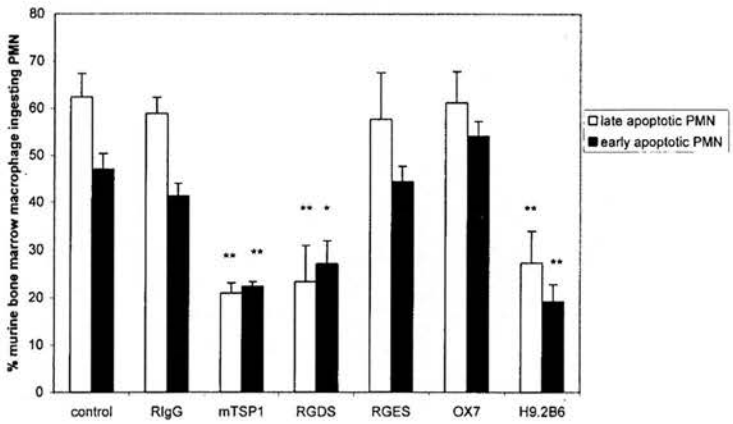

FIGURE 7. BALB/c murine bone marrow-derived Mϕ phagocytosis of aged PMN. Comparison of phagocytosis of late apoptotic PMNs (□) and early apoptotic PMNs (■) prepared from the same 22-h aged human PMN populations. Note that phagocytosis of both apoptotic and late apoptotic PMN was inhibited by rabbit polyclonal Ab to murine TSP1 (mTSP1), H9.2B8 mAb to murine α_v, and RGDS peptide, but not by respective controls, rabbit IgG (RIgG), OX7 mAb, and RGES peptide. Values are the mean ± SE ($n = 6$). *, $p < 0.05$; **, $p < 0.001$.

was ingestion of early apoptotic neutrophils. Furthermore, strong evidence against a requirement for β_2 integrins in efficient Mϕ phagocytosis of either early or late apoptotic neutrophils was provided by the failure of Mϕs from $\alpha_m{}^{-/-}$ and $\beta_2{}^{-/-}$ knockout mice to exhibit any defect in phagocytosis of either target.

Our first major conclusion from these data is that progression beyond the (early) apoptotic state originally defined in our studies of the constitutive death of cultured neutrophils (3) does not deny senescent neutrophils the opportunity for safe clearance while in the late apoptotic state before potentially deleterious secondary necrosis. Although our assessment of the proinflammatory secretory response from Mϕs taking up late apoptotic neutrophils was limited to assay of Mϕ release of the key chemokine IL-8 and the master inflammatory mediator TNF-α, previous studies (11–13) indicate that other classes of inflammatory mediator are unlikely to be released by Mϕ under these circumstances. The current data re-emphasize the tissue-protective potential of neutrophil clearance by apoptosis, demonstrating that even cells reaching what might be termed the "last chapter" of constitutive death can be cleared safely.

However, our data also indicate that it may prove difficult to selectively define the potential for tissue protection afforded by

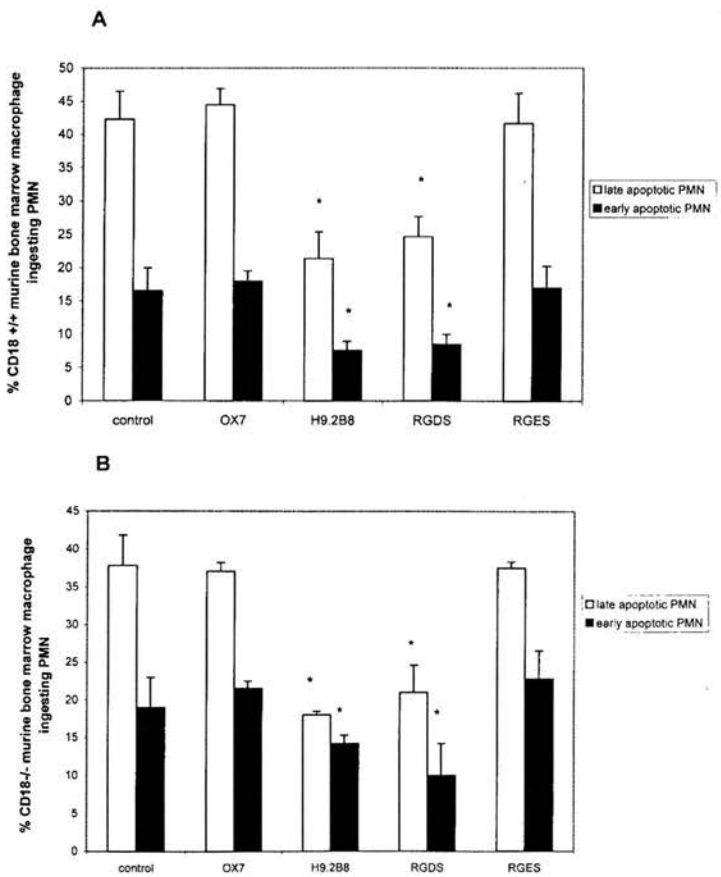

FIGURE 8. Effect of CD18 deficiency on murine bone marrow-derived Mϕ phagocytosis of aged PMN. Comparison of phagocytosis of late apoptotic PMNs (□) and early apoptotic PMNs (■) prepared from the same 22-h aged human PMN populations. *A*, Wild-type control; *B*, Mϕ from $CD18^{-/-}$ knockout mice. Data are the mean ± SE ($n = 3$). *, $p < 0.05$. Note that in both cases there was inhibition of uptake of both types of aged PMNs by 100 μg/ml mAb H9.2B8 and 1 mM RGDS peptide, but not by identical concentrations of controls, mAb OX-7 and RGES peptide.

specific clearance of late apoptotic neutrophils in vivo. This is because Mϕ phagocytosis of both early and late apoptotic neutrophils appears likely to involve similar mechanisms in which TSP1 and the vitronectin receptor play a major role. Thus, it may prove difficult to inhibit uptake of early apoptotic cells selectively, because this would be necessary for a formal assessment of the capacity of Mϕ phagocytosis of late apoptotic neutrophils to serve as a last line of tissue defense in inflammation. Nevertheless, despite a growing understanding of molecular mechanisms mediating phagocytic clearance, we still know very little of those mechanisms that predominate in the clearance of apoptotic cells from various sites in the living mammal. Future work should take into account the possibility that clearance mechanisms dedicated to removal of late apoptotic cells could exist. Some support for this possibility can be drawn from the observation by Rubartelli et al. that myeloid dendritic cells may use α_v integrins in selective ingestion of late apoptotic cells (26).

The second major finding of our study also emphasizes that much remains to be learned about mechanisms mediating Mϕ ingestion of cells dying by apoptosis. The data clearly demonstrate that expression of β_2 integrins is not necessary for efficient phagocytosis of either early or late human apoptotic neutrophils by murine bone marrow-derived Mϕs (which, nevertheless, appear to use TSP1/vitronectin receptor-mediated mechanisms similar to those exhibited by human monocyte-derived Mϕ). The current data are in keeping with our earlier work (3, 38) in an assay system in which neutrophils underwent apoptosis in the presence of autologous PRPDS, a source of complement that, according to a recent report, apoptotic cells may activate so that they become coated with opsonic complement fragments (29). Nevertheless, when such cells were interacted in the absence of added serum with human monocyte-derived Mϕ, no defect in phagocytosis of apoptotic neutrophils was observed despite functionally validated Ab blockade of Mϕ, CR1, CR3, and CR4 receptors (38), nor was any obvious defect in phagocytosis exhibited by monocyte-derived Mϕ prepared from a patient with severe congenital β_2 deficiency (40). However, our findings must be set against 1) growing evidence that the first component of complement, C1q, could bridge apoptotic cells to phagocytes in a manner similar to that proposed for TSP1 (41, 42), and 2) compelling data suggesting that Mϕ receptors for opsonic complement fragments could be important in amplifying efficient phagocytosis of dying cells (17, 29). Nevertheless, it is notable that these latter reports have either used mixed populations of dying cells, including cells in secondary necrosis (17), or have used assays of interaction with Mϕ that include a large tethering element (29) rather than our own extensively validated assay of completed phagocytosis. Further studies will be needed to resolve the importance of complement components in removal of neutrophils at various stages of the apoptotic death program.

To conclude, our studies demonstrate that late apoptotic neutrophils can be ingested by Mϕs via specific mechanisms uncoupled from secretory proinflammatory responses. Such mechanisms can operate efficiently in the absence of Mϕ β_2 integrins, however, emphasizing the need for further characterization of the role of complement components in the safe clearance of cells dying by apoptosis.

Acknowledgments

The following are thanked for helpful discussions and/or the provision of reagents Dr. Eric Brown (Washington University, St. Louis, MO), Drs. Vishva Dixit and Karen O'Rourke (University of Michigan, Ann Arbor, MI), Prof. Mike Horton (University College London Medical School, London, U.K.), and Dr. Alex Law (Medical Research Council Immunochemistry Unit, Oxford, U.K.). Carolyn Gilchrist provided expert secretarial assistance.

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Nephrol Dial Transplant (2002) 17: 697–700

Nephrology Dialysis Transplantation

Editorial Comment

Apoptosis and autoimmunity

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Keywords: apoptosis; autoimmunity; inflammation; lupus; phagocytes

Introduction

During normal tissue homeostasis, the rate of cell death is perfectly balanced by the rate of production of new cells, resulting in a constant cell number. Apoptosis is a critically important mechanism that facilitates deletion of unwanted or damaged cells in various circumstances, including embryogenesis, inflammation and tissue healing. The purpose of this article is to outline a 'darker side' of apoptosis since defects in the apoptotic cell death programme and subsequent clearance of cellular corpses are implicated in the pathogenesis of clinically important autoimmune diseases such as systemic lupus erythematosus (SLE).

What is apoptosis?

Apoptosis is characterized by stereotypical morphological and biochemical changes including the activation of specific intracellular proteolytic enzymes (caspases) that cleave myriad nuclear and cytoplasmic substrates [1,2]. Apoptosis may result from an insufficient supply of survival signals or may be actively induced by various injurious stimuli such as hypoxia, reactive oxygen species, complement attack, nitric oxide, cytokines such as tumour necrosis factor-α or ligation of the Fas (CD95) cell surface death receptor. Apoptosis elicits specific cell surface changes, such as the exposure of phosphatidylserine, normally found on the intracellular aspect of the cell membrane, resulting in the swift uptake and degradation of apoptotic cells either by local resident cells or infiltrating phagocytes. This process is very rapid such that apoptotic cells are conspicuously absent in normal tissues. Furthermore, cell deletion by apoptosis leading to clearance by 'professional' phagocytes such as macrophages is not associated with proinflammatory mediator release but rather causes release of anti-inflammatory agents such as transforming growth factor beta 1 (TGFβ1) [3,5]. The mechanisms whereby macrophages and 'semi-professional' phagocytes (including mesangial cells) recognise and ingest apoptotic cells are complicated and may involve numerous molecules including the vitronectin receptor ($\alpha_v\beta_3$ integrin), CD36, thrombospondin, the phosphatidylserine receptor, the first component of complement C1q, β2 thrombomodulin, class A scavenger receptors, etc. [4] (Table 1).

The role of apoptosis in the maintenance of self tolerance and the regulation of T cell populations

Self tolerance requires the removal or deactivation of autoreactive T cells with specificity to both central (i.e. T cells reacting to self antigen present in the thymus) and peripheral antigens. Within the developing thymus, engagement of autoreactive T cells by self antigen induces apoptosis and deletion of these potentially injurious T cells. Consequently, defects in this process will facilitate the persistence of T cells capable of recognising self and inducing autoimmunity.

However, the entire repertoire of self antigens is not represented within the thymus and therefore additional mechanisms are needed to maintain adequate peripheral self tolerance. Although this process is incompletely understood apoptosis may also be involved. It has been recognised for some time that certain organs such as the eye and the testes exhibit 'immunological privilege'. It is now apparent that this is at least partly due to the expression of Fas ligand by resident tissue cells at these sites. This tissue 'self defense' mechanism results in the induction of apoptosis in infiltrating Fas-bearing lymphocytes following Fas ligation.

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Table 1. Receptors and molecules involved in the recognition and ingestion of apoptotic cells

Phagocyte cell surface	Bridging molecules	Apoptotic cell surface
Phosphatidylserine receptor	Thrombospondin	Phosphatidylserine
CD36	C1q	ICAM-3
Integrins ($\beta_3/\beta_1/\beta_2/\beta_5$)	iC3b	Sugar moieties
CD14	B_2GPI	Thrombospondin binding site
Class A scavenger receptor		C1q binding site
C1q receptor		
B_2GPI receptor		
Lectins		

Note that the receptors utilized may differ between various phagocytic cells and that some of the proposed receptors and molecules involved have not been well characterized (e.g. cell surface lectins that binding sugar moieties on the apoptotic cell surface). Adapted from [4].

Apoptosis also plays a role in the regulation of T cell populations. Clonally expanded populations of activated T cells that have served their functional purpose are rapidly deleted by 'activation induced cell death' (AICD), a process dependent upon ligation of the cell surface Fas death receptor. It is therefore very pertinent that mice with mutations in the genes encoding either Fas or Fas ligand develop autoimmune disease. Depending upon the genetic background, such mice exhibit lymphadenopathy, splenomegaly, autoantibody formation, joint inflammation and glomerulonephritis with features of SLE [6]. Interestingly, early treatment of *gld/gld* mice, deficient in functional Fas ligand, with an agonistic anti-Fas antibody protected mice from the subsequent development of autoimmune disease by a mechanism which presumably involves Fas-dependent deletion of autoreactive lymphocytes [7]. Furthermore, treatment of established autoimmune disease with the same agonistic anti-Fas antibody resulted in a significant improvement in disease pathology [7].

A small number of human patients have been described with mutations in the genes encoding either Fas or Fas ligand. These patients exhibit an autoimmune lymphoproliferative syndrome (ALPS or Canale–Smith syndrome) characterized by lymphadenopathy, splenomegaly and autoantibodies directed at blood components such as erythrocytes and platelets [8]. Affected individuals do not typically develop joint or renal disease, and therefore defects in the Fas death receptor or its ligand do not appear vital to the pathology in these organs but can contribute to autoimmune disease.

Apoptotic cells express potential autoantigens on their cell surface

The origin of the autoantibodies typically present in the sera of patients with autoimmune conditions such as SLE (often directed towards intracellular antigens such as DNA, ribonucleoproteins and nucleosomes) has been perplexing as these autoantigens are normally 'invisible' to the immune system because of their localization within the cell. However, seminal work by Casciola-Rosen *et al.* [9] has indicated that keratinocytes undergoing apoptosis display potential autoantigens upon their cell surface where they are available for interaction with immunologically competent cells. These potential autoantigens on apoptotic cells are likely to have undergone proteolysis by caspases, resulting in the production of immunogenic 'altered self' motifs. Indeed, cleavage of intracellular substrates by the enzyme granzyme B can result in unique modification of potential intracellular autoantigens and raises the possibility that cytotoxic lymphocyte-mediated death of target cells may play a specific role in the development of autoimmunity [10].

Defective clearance of apoptotic cells may induce autoimmunity

Although the surface of apoptotic cells may express potential autoantigens, it is obvious that the majority of the population do not develop autoimmune pathology and therefore apoptosis itself is insufficient to induce autoimmune disease. It is believed that, in normal circumstances, apoptotic cells are rapidly ingested and degraded by phagocytes. Indeed, *in vivo* studies of both renal development and glomerulonephritis indicate that the vast majority of apoptotic cells evident in tissue sections actually lie within other cells [11,12]. This suggests that autoimmunity may be more likely to arise if there is a defect in the clearance of apoptotic cells, and accumulating evidence suggests that this is indeed the case.

For example, experiments involving the injection of normal mice with irradiated syngeneic apoptotic thymocytes resulted in the transient development of antinuclear autoantibodies and anticardiolipin, and anti-ssDNA antibodies, albeit at relatively low levels [13]. Furthermore, these mice also exhibited mild glomerular immunoglobulin deposition. These data indicate that exposure to large numbers of apoptotic cells, which may exceed the phagocytic capacity of the reticuloendothelial system, is able to elicit an autoantibody response.

As indicated previously, multiple macrophage cell surface receptors and bridging molecules may be involved in the recognition and ingestion of apoptotic cells (Table 1). However, the involvement of C1q, the first component of the classical complement pathway, is of particular interest since C1q deficiency is strongly associated with the development of SLE [14]. It is therefore extremely important that mice targeted for the deletion of the C1q gene spontaneously develop both autoantibodies and glomerulonephritis [15]. Indeed, the glomerular inflammation in the C1q-deficient mice is characterized by an impressive

accumulation of apoptotic cells within glomeruli, implying that defective clearance of apoptotic cells is an important aetiological factor in the development of disease [15]. In addition, as in human disease, the genetic background of the C1q-knockout mice plays an important role in modulating disease susceptibility and phenotype. A pathogenetic role of defective apoptotic cell clearance in the development of autoimmunity may also explain why exposure to UV light or intercurrent infections are associated with increased disease activity since they would be predicted to increase the burden of apoptotic keratinocytes or leukocytes requiring phagocytic clearance. Lastly, it is pertinent that monocyte-derived macrophages isolated from patients with SLE exhibit reduced ingestion of apoptotic cells in a quantifiable *in vitro* assay of phagocytosis, suggesting that a defect in apoptotic cell clearance may be relevant to the pathogenesis of SLE in C1q-replete human patients [16].

Biological systems have evolved to ensure that apoptotic cells do not normally undergo secondary necrosis, which would be predicted to increase the likelihood of autoimmune responses. For example, C reactive protein, a teleologically ancient acute phase reactant protein, can bind to the apoptotic cell surface. Bound CRP stimulates activation of the classical pathway of complement, resulting in opsonization of the cell with complement components that can augment apoptotic cell clearance [17], and also assists the maintenance of cell viability by inhibiting the activation of the cytolytic C5b-9 terminal membrane attack complex. In addition, the pentraxin PTX3 is an acute phase protein generated locally within inflamed tissues that acts to inhibit uptake of apoptotic cells by dendritic cells, which are capable of presenting apoptotic cell-derived antigen to T cells [18,19]. Finally, the acute phase protein serum amyloid P component (SAP) binds chromatin on apoptotic cell surfaces as well as binding, solubilizing and regulating the degradation of free chromatin that results from cell breakdown. The importance of this chromatin scavenging system is underscored by the phenotype of mice targeted for the deletion of the SAP gene, which exhibit antinuclear autoantibodies and glomerulonephritis [20].

Immunological presentation of antigens derived from apoptotic cells

Unlike macrophages, dendritic cells are unique in their ability to stimulate primary immune responses. Immature dendritic cells exist in the periphery and constantly sample their antigenic milieu. In order to become fully functional antigen-presenting cells they must mature and migrate to draining lymph nodes where they can interact with naïve T cells. Dendritic cell maturation is normally provoked by inflammatory stimuli such as TNF-α or bacterial products. Recent data indicate that dendritic cells not only ingest apoptotic cells but are capable of presenting apoptotic cell-derived antigen to both CD4 and CD8 T cells if they receive appropriate activation signals [18,19]. Since apoptotic cells are a source of potential autoantigens it is conceivable that the phagocytic immature dendritic cell can induce a primary autoimmune response if it encounters apoptotic cells concurrently with appropriate 'danger' signals. Indeed, it has been suggested that a large load of apoptotic cell material, perhaps due to secondary necrosis following failed clearance, may predispose to the development of autoimmune responses by increasing the potential for dendritic cells to phagocytose apoptotic cell material and subsequently present autoantigens [21]. However, this is a controversial area of research as conflicting data suggests that dendritic cells that have ingested apoptotic cells may play a role in the maintenance of peripheral tolerance [22].

Resolution of inflammation and scarring

Defective clearance of apoptotic cells in patients with SLE may actually represent a 'double whammy', i.e. it may promote the development of autoimmunity

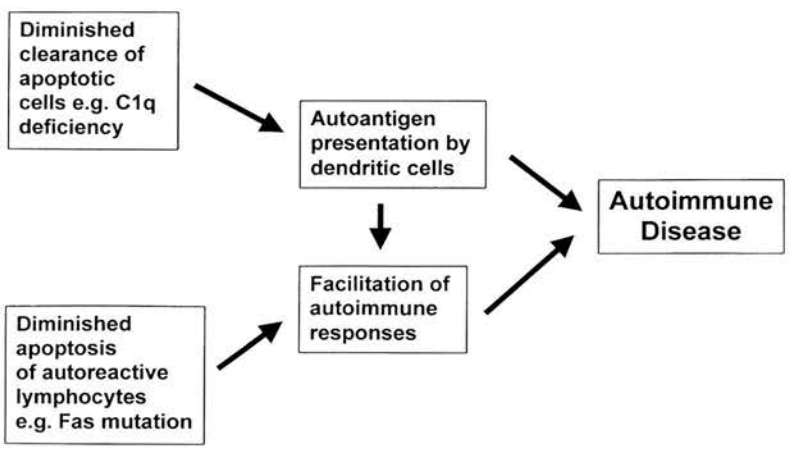

Fig. 1. Simplified schema indicating the role of apoptosis in autoimmunity.

as well as blunt the macrophage 'deactivating' effects of apoptotic cell ingestion at inflamed sites [5]. In theory, this failure to 'switch off' activated pro-inflammatory macrophages may impair the resolution of inflammation in the kidney and other tissues, with the consequent promotion of scarring and loss of organ function.

Conclusion

Apoptosis and the clearance of apoptotic cells are essential for normal tissue homeostasis, embryogenesis and tissue remodelling. Apoptotic deletion of potentially autoreactive T cells is involved in the maintenance of self tolerance. Furthermore, apoptotic cells are an important and preferential source of many potential autoantigens. Defective apoptotic cell clearance may facilitate the inappropriate presentation of potential autoantigens by dendritic cells, thereby initiating autoimmune responses. These observations confirm an important role for defects in apoptosis and the clearance of apoptotic cells in the development of autoimmunity (Figure 1). Future research will shed further light upon the involvement of apoptosis in autoimmunity and hopefully highlight new targets for potential therapeutic intervention.

Acknowledgements. L.S. is in receipt of a Wellcome Trust Clinical Training Fellowship (Grant No. 34842). J.H. is a Wellcome Trust Senior Research Fellow in Clinical Science (Grant No. 061139). We are grateful to Professor John Savill for helpful comments.

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